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(57) Abstract

The present invention relates, in general, to a method of modulating physiological and pathological processes and, in particular, to a method of modulating intra- and extracellular levels of oxidants and thereby processes in which such oxidants are a participant. The invention also relates to compounds and compositions suitable for use in such methods.

OXIDANT SCAVENGERS

This is a continuation-in-part Application No.
08/613,418, filed March 11, 1996, which is a
continuation-in-part of Application No. 08/476,866 filed
5   June 7, 1995, which is a continuation-in-part of
Application No. 08/322,766 filed October 13, 1994, which
is a continuation-in-part of Application No. 08/136,207,
filed October 15, 1993, the contents of these
applications being incorporated herein by reference.

10   TECHNICAL FIELD

The present invention relates, in general, to a
method of modulating physiological and pathological
processes and, in particular, to a method of modulating
intra- and extracellular levels of oxidants such as
15   superoxide radicals and hydrogen peroxide and thereby
processes in which such radicals are a participant. The
invention also relates to compounds and compositions
suitable for use in such methods.

BACKGROUND

20   Oxidants are produced as part of the normal
metabolism of all cells but also are an important
component of the pathogenesis of many disease processes.
Reactive oxygen species, for example, are critical
elements of the pathogenesis of diseases of the lung,
25   the central nervous system and skeletal muscle. Oxygen

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free radicals also play a role in modulating the effects
of nitric oxide (NO·). In this context, they contribute
to the pathogenesis of vascular disorders, inflammatory
diseases and the aging process.

5   A critical balance of defensive enzymes against
oxidants is required to maintain normal cell and organ
function. Superoxide dismutases (SODs), a family of
metalloenzymes which catalyze the intra- and
extracellular conversion of O_2^- into H_2O_2 plus O_2, and

10   represent the first line of defense against the
detrimental effects of superoxide radicals. Mammals
produce three distinct SODs. One is a dimeric copper-
and zinc-containing enzyme (CuZn SOD) found in the
cytosol of all cells. A second is a tetrameric

15   manganese-containing SOD (Mn SOD) found within
mitochondria, and the third is a tetrameric,
glycosylated, copper- and zinc-containing enzyme
(EC-SOD) found in the extracellular fluids and bound to
the extracellular matrix. Several other important

20   antioxidant enzymes are known to exist within cells,
including catalase and glutathione peroxidase. While
extracellular fluids and the extracellular matrix
contain only small amounts of these enzymes, other
extracellular antioxidants are known to exist, including

25   radical scavengers and inhibitors of lipid peroxidation,
such as ascorbic acid, uric acid, and α-tocopherol
(Halliwell et al, Arch. Biochem. Biophys. 280:1 (1990)).
The relative lack of extracellular antioxidant enzymes
may reflect the possible function of extracellular

30   reactive oxygen species as bioeffector molecules

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(Halliwell et al, Arch. Biochem. Biophys. 280:1 (1990)).
The relative deficiency of such enzymes may also result
in greater susceptibility to extracellular oxidant
stresses.

5   The enzyme EC-SOD, in many extracellular locations,
exists only at low concentrations. While its
physiologic role *in vivo* is yet to be defined, in many
extracellular locations, EC-SOD is not thought to
function as a bulk scavenger of O_2^-. As indicated
10   above, EC-SOD is a tetrameric Cu/Zn-containing
glycoprotein with a subunit molecular weight of 30,000
(Marklund, Proc. Natl. Acad. Sci. USA 79:7634 (1982);
Tibell et al, Proc. Natl. Acad. Sci. USA 84:6634 (1987);
see also USP 5,130,245 and WO 91/04315). Biochemical
15   data suggest that EC-SOD binds to heparan sulfate
proteoglycans on endothelial cells, where it has been
speculated to serve as a "protective coat" (Marklund, J.
Clin. Invest. 74:1398 (1984); Karlsson et al, Biochem.
J. 255:223 (1988)). Endothelial cells secrete both O_2^-
20   (Halliwell, Free Radical Res. Commun. 5:315 (1989)) and
endothelium-derived relaxing factor, putatively
identified as nitric oxide (NO·) (Noak and Murphy, *in*
Oxidative Stress Oxidants and Antioxidants, eds Sies, H.
(Academic, San Diego), pp. 445-489 (1991)). NO·
25   functions as a vasoregulator and as a regulator of
neurotransmission (Schuman and Madison, Science 254:1503
(1991)). NO· can, however, be toxic to neurons in some
situations (Dawson et al, Proc. Natl. Acad. Sci. USA
88:6368 (1991)). O_2^- is known to inactivate NO·-induced
30   vasorelaxation (Gryglewski et al, Nature 320:454 (1986);

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Rubanyi and Vanhoutte, Am. J. Physiol. 250:H822 (1986);
Rubanyi and Vanhoutte, Am. J. Physiol. 250:H815 (1986);
Bult et al, Br. J. Pharmacol. 95:1308 (1988); Nucci et
al, Proc. Natl. Acad. Sci. USA 85:2334 (1988)). Thus, a
5   possible function for EC-SOD is to protect NO· released
from cells from O_2^--mediated inactivation.

The reaction of O_2^- with NO· is also known to
produce a potentially toxic intermediate in the form of
the peroxynitrite anion (ONOO⁻) (Beckman et al, Proc.
10   Natl. Acad. Sci. USA 87:1620 (1990); Mulligan et al,
Proc. Natl. Acad. Sci. USA 88:6338 (1991); Hogg et al,
Biochem. J. 281:419 (1992); Matheis et al, Am. J.
Physiol. 262:H616 (1992)). Thus EC-SOD may also
function to prevent the formation of ONOO⁻.

15   Surprisingly, it has been found that EC-SOD
increases, rather than decreases, central nervous system
O_2 toxicity and that this effect of EC-SOD occurs
through modulation of NO·. This result implicates NO·
as an important mediator in O_2 toxicity. The invention
20   thus relates to methods of manipulating nitric oxide
function that involve the use of extracellular anti-
oxidants.

In addition to superoxide radicals, hydrogen
peroxide is an oxidant species that is produced under a
25   wide variety of conditions of oxidant stress. The
invention thus also provides a method of manipulating
hydrogen peroxide levels.

The methods of the invention find application in
various disease and non-disease states in which
30   oxidative stress plays a role, including inflammation.

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In a broader sense, the invention relates generally to
methods of modulating intra- and extracellular processes
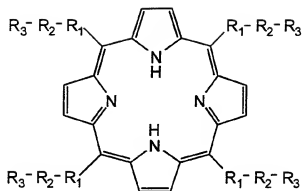in which an oxidant such as O_2^- or hydrogen peroxide is
a participant.

5   SUMMARY OF THE INVENTION

        The present invention relates to a method of
modulating intra- or extracellular levels of oxidants
such as superoxide radicals, hydrogen peroxide and
peroxynitrite. More particularly, the invention relates
10   to a method of modulating normal or pathological
processes involving superoxide radicals, hydrogen
peroxide, nitric oxide or peroxynitrite using low
molecular weight antioxidants, for example, mimetics of
SOD, catalase or peroxidase.

15   In one embodiment, the present invention relates to
an oxidant scavenger, for example, a mimetic of
superoxide dismutase, catalase or peroxidase, comprising
a nitrogen-containing macrocyclic moiety and a cell
surface or extracellular matrix targeting moiety, or a
20   pharmaceutically acceptable salt thereof.

        In another embodiment, the present invention
relates to oxidant scavenger of the formula:

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or a pharmaceutically acceptable salt thereof, wherein:

R_1 is a bond, , , , ,
, , , or wherein X is a

halogen and Y is an alkyl group and wherein

indicates bonding to R_2 at any position and

indicates bonding to R_2 and the substituent at any

position; and

R_2 is a bond, $-(CY_2)_n^-$, $-(CY'_2-CY'=CY')_n^-$, $-(CY'_2-$
$CY'_2-CH=CH)_n^-$, $-(CY'=CY')_n^-$, or $-(CY'_2-\overset{O}{\underset{H}{C}})_n^-$, wherein Y'

is hydrogen or an alkyl group and wherein n is 1 to 8;

and

R_3 is $-Y''$, $-OH$, $-NH_2$, $-N^+(Y'')_3$, $-COOH$, $-COO^-$,

$-SO_3H$, $-SO_3^-$, $-C-PO_3H_2$ or $-C-PO_3H^-$, wherein Y'' is an

alkyl group,

wherein, when R_1 is and R_2 is a bond, R_3 is

not Y'' (eg $-CH_3$), and

wherein, when R_1 is and R_2 is a bond, R_3 is

not $-Y''$ (eg $-CH_3$), $-N^+(Y'')_3$ (eg $-N^+(CH_3)_3$), or COOH.

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SUBSTITUTE SHEET (RULE 26)

In a further embodiment, the present invention relates to an oxidant scavenger of the formula:

or a pharmaceutically acceptable salt thereof, or metal complex thereof wherein said metal is selected from the group consisting of manganese, copper and iron, wherein:

each R_1' is independently a bond, , ,

 , , , , , or

 , wherein Y" is an alkyl group, and wherein

indicates bonding to R_2' at any position and

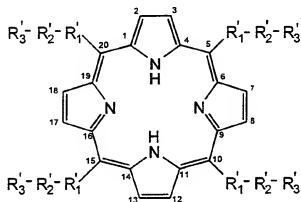indicates bonding to R_2' and the R_1' phenyl substituent

at any position;

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each R_2' is independently a bond, or $-(CH_2)_n-$
wherein n is 1-4,

each R_3' is independently $-Y''$, $-Y'''$, $-H$, $-OH$, $-OY''$,
$-NO_2$, $-CN$, $-NH_2$, $-COOH$, $-COY''$, $-COO^-$, or a heterocyclic
group, wherein Y'' is as defined above and Y''' is a
primary, secondary, tertiary or quaternary amine,

wherein when R_1' is , R_3' is not COOH, COY''
or COO^-,

wherein when R_1' is , , or ,
R_3' is not $-NO_2$, and wherein $-R_1'-R_2'-R_3'$, collectively,
are not $-H$, , , , ,
or .

In yet a further embodiment, the present invention
relates to a method of protecting cells from superoxide
radical-, hydrogen peroxide- or peroxynitrite-induced
toxicity comprising contacting the cells with an oxidant
scavenger, eg a SOD, catalase or peroxidase mimetic,
sufficient to effect the protection.

In another embodiment, the present invention
relates to a method of inhibiting damage due to
oxidation of a substance, for example, with the subse-
quent formation of O_2^-, hydrogen peroxide, or peroxy-
nitrite comprising contacting the substance with an

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amount of a SOD mimetic sufficient to effect the
inhibition.

In a further embodiment, the present invention
relates to a method of inhibiting xanthine oxidase
5   activity of a cell or tissue comprising contacting the
cell or tissue with an amount of an oxidant scavenger
sufficient to effect the inhibition.

In another embodiment, the present invention
relates to a method of treating a pathological condition
10   of a patient (eg, of the lungs of a patient) resulting
from superoxide radical-induced degradation of NO·. The
method comprises administering to the patient (eg, to
the airways of the patient) an effective amount of a
compound having the activity and tissue specificity of
15   SOD (eg EC-SOD) under conditions such that the treatment
is effected.

In a further embodiment, the present invention
relates to a method of treating an inflammatory
condition in a patient in need of such treatment
20   comprising administering to the patient an effective
amount of an oxidant scavenger, eg a mimetic of SOD, (eg
EC-SOD), catalase or peroxidase, under conditions such
that the treatment is effected.

In another embodiment, the present invention
25   relates to a method of treating a disorder resulting
from aberrant smooth muscle function in a patient in
need of such treatment comprising administering to the
patient an effective amount of a mimetic of SOD (eg
EC-SOD) under conditions such that the treatment is
30   effected.

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In a further embodiment, the present invention relates to a method of modulating physiologic functions of NO· in a mammal comprising administering to the mammal an oxidant scavenger in an amount sufficient to effect the modulation. Physiologic functions of NO· include its function as a smooth muscle relaxant, neurotransmitter and immune modulator.

In yet a further embodiment, the present invention relates to soluble oxidant scavengers, for example, mimetics of SOD, catalase or peroxidase, and to targeted forms thereof, in particular, mimetics of EC-SOD having a GAG binding moiety attached thereto.

In another embodiment, the present invention relates to an isolated EC-SOD gene sequence, or portion thereof.

Objects and advantages of the present invention will be clear from the description that follows.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the EC-SOD expression vector used to construct transgenic mice. Transgenic mice were generated with the Eco-RI-XbaI fragment. IVS1: Intervening sequence 1 from the human ß-actin promoter.

Figure 2 shows the Northern analysis of tissues from transgenic mice. Twenty μg of total RNA from the tissues of transgenic mice were denatured with glyoxal and electrophoresed through a 1.2% agarose gel and blotted onto nitrocellulose. The filter was probed with

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the entire human EC-SOD cDNA. The 2.5 Kb band
corresponds to mRNA of the human EC-SOD transgene
containing the 1 Kb intervening sequence (see Figure 1).
The 1.5 Kb band corresponds to the fully processed mRNA
5   of the human EC-SOD transgene.

    Figure 3 shows the percent survival of transgenic
and nontransgenic mice exposed to 6 ATA oxygen for
25 minutes. Mice were injected with saline or given
20 mg/kg N-ω-nitro-L-arginine (LNNA) i.p. 10 minutes
10   before compression. 400 mg/kg of diethyldithiocarbamate
(DDC) in saline was injected i.p. 55 min before
compression. *$p < 0.017$ tested by χ^2 with Bonferroni
correction, compared to transgenic saline treated mice.

    Figure 4 shows time to onset of first seizure in
15   transgenic and nontransgenic mice exposed to 6 ATA
oxygen. Mice were injected with saline or given
20 mg/kg N-ω-nitro-L-arginine (LNNA) i.p. 10 minutes
before beginning compression. 400 mg/kg diethyldithio-
carbamate (DDC) was injected i.p. 55 minutes prior to
20   compression. Results are expressed as mean ± S.D. of
time to first seizure with zero time taken once chamber
reached 6 ATA. *$p < 0.05$ tested by analysis of variance
with the Scheffe F-test compared to nontransgenic saline
treated mice.

25   Figure 5 shows the effect of diethyldithiocarbamate
and ß-mercaptoethanol on survival in 6 ATA oxygen for
30 minutes. (C57BL/6 X C3H)F1 mice were injected i.p.

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with saline, 180 mg/kg ß-mercaptoethanol (2-ME), or
400 mg/kg diethyldithiocarbamate (DDC) in saline 55 min.
before compression. *p<0.025 tested by χ2 with
Bonferroni correction compared to saline treated mice.

5   Figure 6 shows the seizure latency in wild-type
mice exposed to 6 ATA oxygen after being treated with
saline or 20 mg/kg N-ω-nitro-L-arginine (LNNA) or
20 mg/kg N-ω-nitro-L-arginine plus 50 mg/kg L-arginine
(LNNA + L-Arg). *p<0.05 tested by analysis of variance
10   with a paired Student's t-test compared to saline
treated mice.

     Figure 7 shows the percent survival in wild-type
mice exposed to 6 ATA oxygen. Mice were given an i.p.
injection of normal saline (0.008 cc/g) or 20 mg/kg
15   N-ω-nitro-L-arginine (LNNA) (0.008 cc/g) 15 minutes
prior to compression. The mice were exposed to 6 ATA of
oxygen for 20 minutes (n=10, saline only), 25 minutes
(n=10, both groups), 30 minutes (n=10, saline only),
50 minutes (n=6, LNNA only), 75 minutes (n=12, LNNA
20   only), 90 minutes (n=14, LNNA only), 105 minutes (n=6
LNNA only) and 120 minutes (n=6, LNNA only) and percent
survival was measured for each group.

     Figure 8 shows the survival dose response curve for
N-ω-nitro-L-arginine (LNNA). Wild-type mice were given
25   an i.p. injection of normal saline (0.008 cc/g) or 0, 2,
10, 20, or 30 mg/kg LNNA (0.008 cc/g) 15 minutes prior
to compression and then exposed to 75 minutes at 6 ATA

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oxygen. Percent survival was calculated for each
treatment group.

     Figure 9 shows the percent survival in wild-type
mice pretreated with saline, 20 mg/kg N-ω-nitro-L-
arginine (LNNA), or 20 mg/kg N-ω-nitro-L-arginine plus
50 mg/kg L-arginine (LNNA + L-Arg) and then exposed to
75 minutes of 6 ATA oxygen. *$p < 0.05$ tested with a χ-
square test with Bonferroni correction.

     Figure 10 shows the percent survival in transgenic
and nontransgenic mice exposed to 6 ATA oxygen for
75 minutes. Mice were injected with saline or given
20 mg/kg N-ω-nitro-L-arginine (LNNA) i.p. 10 minutes
before compression. *$p < 0.05$ tested by χ^2 compared to
nontransgenic saline treated mice. $+$ $p < 0.05$ tested by
χ^2 compared to transgenic saline treated mice.

     Figure 11 shows the comparison of edema formation
in EC-SOD transgenic mice to edema formation in
nontransgenic littermates after cold-induced injury to
the right cerebral hemisphere as well as in non-injured
mice. Values are presented as mean ± standard error.
*$p < 0.05$ compared to Edema Index of respective
nontransgenic controls using a paired Student's t-test.

     Figure 12 shows the effect of augmented levels of
EC-SOD on vascular permeability changes after cold-
induced brain injury. Vascular permeability is demon-
strated as Evan's blue leakage in the injuried right

                              13

cerebral hemispheres of nontransgenic (control) and EC-
SOD transgenic mice.

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Figure 13 shows a Western blot analysis of
rh-EC-SOD and a human lung homogenate to demonstrate
antibody specificity. Proteins were separated on a 10%
0.75 mm SDS-polyacrylamide gel and transferred to nitro-
cellulose. Proteins were hybridized with the antibody
to recombinant human EC-SOD (4.3 μg/ml) and the antibody
was detected by hybridization with ^{125}I-Protein-A

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followed by autoradiography. The EC-SOD lane contained
0.05 μg of pure recombinant human type C EC-SOD lane
protein. The lung lane contained 10 μg of a 20,000 x g
supernatant of a human lung homogenate.

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Figures 14A-14C show the light microscopic
immunohistochemical localization of EC-SOD in human
lung. Tissues were labeled using the antibody to
recombinant human EC-SOD (5.4 mg/ml; anti-EC-SOD) or the
same antibody in which the anti-EC-SOD IgG was absorbed
out using purified recombinant EC-SOD attached to CNBr-

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sepharose (EC-SOD absorbed). Antibody was detected
using a biotin/streptavidin-hoseradish peroxidase
labeling technique. A, Large elastic pulmonary artery
labeled with anti-EC-SOD. Note labeling around smooth
muscle cells beneath the endothelium and beneath the

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elastic layer of the vessel (short arrow), and the lack
of labeling for EC-SOC on the surface of endothelial
cells (open arrow) and on elastin (long arrow). B,
Muscular pulmonary artery labeled with anti-EC-SOD.

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Note high amount of labeling in the connective tissue
matrix surrounding the vessel and lymphatics (long
arrow), in the matrix surrounding smooth muscle cells
(short arrow), and the lack of labeling on the surface
5   of endothelial cells (open arrow). C, Muscular
pulmonary artery labeled with EC-SOD absorbed antisera.
The absorption of anti-EC-SOD IgG abolished all labeling
in the muscular vessel.   (Bars = 50 μm).

      Figures 15A-15C show the immunohistochemical
10   localization of EC-SOD in human lung. Tissues were
labeled using the antibody to recombinant human EC-SOD
(5.4 mg/ml; anti-EC-SOD). Antibody was detected using a
biotin/streptavidin-horseradish peroxidase labeling
technique. A, Large cartilaginous airway labeled with
15   anti-EC-SOD. Note the intense labeling for EC-SOD in
the matrix around smooth muscle cells (short arrow),
between the epithelial cells (long arrow), and the lack
of labeling on the surface of the epithelial cells (open
arrows), and in the matrix of cartilage (asterisk). B,
20   Noncartilaginous airway labeled with anti-EC-SOD. Note
the intense labeling for EC-SOD throughout the entire
matrix beneath the epithelium (short arrow), and the
lack of labeling on the surface of the epithelium (open
arrow). C, Lung parenchyma labeled with anti-EC-SOD.
25   EC-SOD labeling is primarily at alveolar septal tips
(short arrow), and in the matrix surrounding small
vessels (long arrow). No labeling for EC-SOD was seen
on the surface of alveolar epithelial cells (open
arrow). (Bars = 50 μm).

15

Figures 16A-16C show the electron microscopic
immunolocalization of EC-SOD in vascular connective
tissue. Tissues were labeled using the antibody to
recombinant human EC-SOD (40 μg/ml; anti-EC-SOD) or the
5   same antibody after the anti-EC-SOD IgG was absorbed out
using purified recombinant EC-SOD attached to CNBr-
sepharose (EC-SOD absorbed). Antibody was detected
using 10 nm protein-A gold. A, Vascular collagen
labeled with anti-EC-SOD, B, Vascular elastin labeled
10   with anti-EC-SOD. C, Vascular collagen labeled with
EC-SOD absorbed antisera. Note the intense labeling of
EC-SOD in association with type I collagen and the lack
of labeling in association with elastin (E). In
addition, absorption of anti-EC-SOD antibody abolished
15   all labeling for EC-SOD in association with type I
collagen. (Bars = 200 nm).

Figure 17 shows the electron microscopic immuno-
localization of EC-SOD around vascular smooth muscle.
Tissues were labeled using the antibody to recombinant
20   human EC-SOD (40 μg/ml). Antibody was detected using
10 nm protein-A gold. There is a high degree of
labeling in the connective tissue matrix around the
vascular smooth muscle cell (S) in association with
type I collagen (short arrow), and other unidentified
25   matrix elements (long arrow). (Bars = 200 nm).

Figures 18A-18B show the electron miscropic immuno-
localization of EC-SOD on the surface of pulmonary
endothelial cells. Tissues were labeled using the

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antibody to recombinant human EC-SOD (40 μg/ml).
Antibody was detected using 10 nm protein-A gold. A,
Endothelial cell from a small muscular pulmonary artery,
B, Endothelial cell from a pulmonary capillary. No
5   labeling for EC-SOD was on the surface of the
endothelial cells (short arrows). EC-SOD is seen in the
plasma (P) and is associated with extracellular matrix
proteins beneath the endothelium (long arrows).
(Bars = 200 nm).

10   Figure 19 shows the electron microscopic immuno-
localization of EC-SOD around bronchial epithelial
cells. Tissues were labeled using the antibody to
recombinant human EC-SOD (40 μg/ml). Antibody was
detected using 10 nm protein-A gold. EC-SOD was found
15   in the junction between the epithelial cells (arrow) and
was also seen to some extent inside the cells.
(Bars = 200 nm).

Figures 20A-20D show a partial restriction map,
sequencing strategy, genomic structure, and protein
20   structure of human EC-SOD Clone #7. Fig. 20A, a partial
restriction map of human EC-SOD genomic clone #7 is
shown in the 5' to 3' orientation. A 1 kb size marker
is indicated. B, BamH I; H, Hind III; P, Pst I; S,
Sal I; K, Kpn I; E, EcoR I. In Fig. 20B, the subcloning
25   and sequencing strategy is shown. Various size
overlapping restriction fragments were subcloned into
the plasmid vector pGEM3Zf(+) for subsequent DNA
sequence analysis. All DNA was sequenced on both

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strands using Sequenase (USB) and double-stranded DNA
template, except for ~2 kb of the 3' 7K36 fragment in
which only one orientation was sequenced. In Fig. 20C,
the exon/intron structure of the human EC-SOD gene is

5   shown. The position of the coding region for preEC-SOD
in exon 3 is shown by the dashed lines. In Fig. 20D,
the four structural domains of human EC-SOD protein are
diagrammed. The signal peptide is indicated by an
arrow. This is followed by the mature glycosylated

10   (CHO) amino terminal peptide domain. A third region has
very high amino acid sequence homology to human CuZn-
SOD. The carboxy terminal domain has multiple charged
basic residues (+) which are critical for binding
heparin glycosaminoglycan.

15   Figures 21A-21B show human multiple tissue Northern
blots of EC-SOD. Fig. 21A, two μg of poly A(+) mRNA
from eight different human tissues were electrophoresed
on a denaturing agarose gel, transferred to a charged
nylon membrane, and probed with [32P]-labeled antisense

20   human EC-SOD cRNA. RNA molecular size markers
(kilobases) are shown on the right. Quantitative
transfer was monitored by ethidium bromide staining.
The results demonstrate a unique 1.4 kb mRNA present in
all eight tissues examined. Interestingly skeletal

25   muscle demonstrates a second, larger mRNA of ~4.2 kb,
while brain shows a faint approximately 2.2 kb band. In
Fig. 21B, bands corresponding to EC-SOD mRNA were
quantitated by laser densitometric scanning, normalized

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to the 1.4 kb brain band, and expressed as relative
absorbance units.

Figures 22A-22B show analysis of the transcription
initiation site. The 5' rapid amplification of cDNA

5   ends (5' RACE) technique was used to identify the site
of transcription initiation for the human EC-SOD gene.
In Fig. 22A, a schematic diagram illustrates the
annealing sites for the various oligonucleotides. The
dark line represents first-strand reverse transcribed

10   cDNA of human heart poly A(+) mRNA which has been primed
with EC7 (an EC-SOD gene specific primer) and poly C
tailed using terminal deoxynucleotidyl transferase
(TdT). HEC1, HEC2, EC4, and EC7 are 5' human EC-SOD
gene specific primers. The anchor primer is supplied

15   with the 5' RACE kit (GIBCO BRL) and hybridized to the
poly C tail. In Fig. 22B, PCR was used to amplify
segments of DNA using [anchor + EC4] or [HEC1 + EC7] as
primers and either poly C tailed (+TdT, lanes 1 & 4) or
non-poly C tailed (-TdT, lanes 2 & 5) cDNA as template.

20   Lane 3 includes PCR amplified DNA using [HEC1 + EC7] as
primers and a full-length human EC-SOD cDNA as template.
The resulting amplified DNAs were electrophoresed on a
2% agarose gel, transferred to charged nylon membranes,
and probed with [^{32}P]-labeled HEC2, a 5' nested gene

25   specific EC-SOD primer. DNA molecular weight markers
were run between lanes 2 and 3. The expected size of
the PCR amplified region in lanes 3, 4 and 5 is 217 bp.
Only a single band is seen in lane 1, with a molecular
size of approximately 185 to 200 bp.

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Figure 23 shows genomic Southern blot analysis of
the human EC-SOD gene. Ten micrograms of human genomic
DNA were completely digested with each of the
restriction enzymes shown, electrophoresed on a 1%
5   agarose gel and transferred to charged nylon membranes.
The blots were proved with a [^{32}P]-labeled EC-SOD
partial length cRNA which corresponds to the first
approximate 1050 nucleotides and autoradiographed. The
specific restriction endonuclease is shown at the top of
10   each lane. DNA molecular size markers (in kilobases)
are shown on the right.

Figure 24 shows the nucleotide sequence and deduced
amino acid sequence of the human EC-SOD gene. The
complete nucleotide sequence of the human gene is shown.
15   The deduced amino acid sequence of the signal peptide
and mature protein is indicated using the single letter
amino acid code.

Figure 25 shows a Lineweaver-Burk plot
demonstrating non-competitive inhibition of xanthine
20   oxidase by MnTBAP.

Figure 26 shows the protection of pulmonary artery
endothelial cells from xanthine oxidase-induced injury
by MnTBAP. Control ▔; MnTBAP ▨.

Figure 27 shows the protection of lung epithelial
25   cells from paraquat-induced injury of SOD mimetics.

20

Figure 28 shows the protection of pulmonary artery endothelial cells from paraquat-induced injury by MnTBAP.

Figure 29 shows the lack of protection of pulmonary artery endothelial cells from paraquat-induced injury by ZnTBAP.

Figure 30 shows the protection of MnTBAP against paraquat-induced lung injury.

Figure 31 shows a second order rate constant plot for catalase mimetics.

Figure 32 shows the effect of MnTBAP on H_2O_2-induced endothelial injury.

Figures 33A and 33B show the reduction by MnTBAP and MnTmPyP of endothelial cell injuries caused by exposure to glucose oxidase-produced hydrogen peroxide. Figure 33C shows that ZnTBAP does not reduce hydrogen peroxide-induced endothelial cell injury. Figure 33D shows that endothelial cells are not protected from hydrogen peroxide-induced injury by CuZnSOD.

Figures 34A and 34B show the time course of NMDA- and KA-induced inactivation of aconitase. Fig. 34A. Cortical cells treated with vehicle or 50 μM NMDA for 0, 5, 15, 30, 60 and 240 min. and aconitase activity measured in cell lysates. Each point represents the mean

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± S.E.M. (n=6-8). Fig. 34B. Cortical cells treated
with vehicle or 300 μM kainate for 0, 60, 240, and 360
min. and aconitase activity measured in cell lysates.
Each point represents the mean ± S.E.M. (n=4-8).

5   Figures 35A-35C show the correlation of toxicity
with aconitase inactivation. Concentration-dependence
of KA (Fig. 35A), PQ⁺⁺ (Fig. 35B) and NMDA (Fig. 35C) to
induce toxicity (left axis) and aconitase inactivation
(right axis) were determined. Values normalized as a
10 percent LDH release or aconitase inhibition and plotted
to assess the correlation between LDH release and
aconitase inactivation. Closed squares represent LDH and
open squares represent aconitase. Curves were computer-
generated using non-linear regression analysis using the
15 equation $Y= Bottom + (Top-Bottom)/1+10^{LogEC50-X}$ (GraphPad
Prism). Each point represents the mean value (n= 4-6).

      Figures 36A and 36B show the blockade of aconitase
inactivation and neurotoxicity by MnTBAP. Fig. 36A.
Cortical cells were treated with 150 μM PQ⁺⁺ for 3 hr.
20 (solid bars), 50 μM NMDA for 1 hr. (open bars) or 300 μM
KA for 6 hr. (hatched bars) in the presence or absence
of 200 μM MnTBAP (present 15 min. prior to and
throughout the duration of treatment) and aconitase
activity measured in cell lysates. Bars represent mean
25 ± S.E.M. (n=8-12). Asterisk indicates a difference from
all other treatments (p<0.05, one-way ANOVA). Fig. 36B.
Cortical cells were treated with 150 μM PQ⁺⁺ (solid
bars), 50 μM NMDA (open bars) or 300 μM KA (hatched

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bars) in the presence of varying concentrations of
MnTBAP (present 15 min. prior to and throughout the
duration of treatment) for 18 hr. and LDH release
measured in the medium. Asterisk represents a

5   difference from controls (agonist in the absence of
MnTBAP; p< 0.05 Dunnet's test). Bars represent mean $\pm$
S.E.M. (n=3-6).

     Figure 37 shows the inhibition of NMDA toxicity by
MnTBAP. Cortical cells were treated with varying

10   concentrations of NMDA in the presence and absence of
200 μM MnTBAP for 18 hr. and LDH measured in media. Each
points represents mean $\pm$ S.E.M., n=3-4.

     Figures 38A-38C show the differential effect of
MnTBAP and ZnTBAP on cell death. Cortical cells were

15   treated with 150 μM PQ^{++} (Fig. 38A), 50 μM NMDA (Fig.
38B) or 300 μM KA (Fig. 38C) in the presence or absence
of varying concentrations of MnTBAP (open squares) or
ZnTBAP (closed squares) and dead cells stained with
EthD-1. Images of EthD-1 -positive cells were stored

20   and counted in randomly selected fields using a digital
image analyzer. Data are expressed as the number of dead
cells per field. Each point represents measurements made
from 1200-1500 cells.

     Figure 39 shows the effect on learning of removal

25   of the EC SOD gene in a mouse model.

Figure 40. Effect of Mn(III)tetrakis (4-benzoic acid) porphyrin (MnTBAP) (3-100 μM) on the oxidation of dihydrorhodamine 123 to rhodamine 123 in response to peroxynitrite (5 μM). Data are expressed as means $\pm$

5   s.e.m. of triplicate determinations.

Figure 41. Suppression by the peroxynitrite (Fig. 41A) and the NO donor compounds S-nitroso-N-acetyl-DL-penicillamine (SNAP, 3 mM) (Fig. 41B) and diethylamine:NO NONOate (DNO) (Fig. 41C) on

10   mitochondrial respiration (expressed as percent of respiration of unstimulated cells) in J774 macrophages, and the protective effect of Mn(III)tetrakis (4-benzoic acid) porphyrin (MnTBAP) (10-300 μM) against this suppression. Data are expressed as means $\pm$s.e.m. of

15   n=12 wells. [**]$p<0.01$ represents significant effect of SNAP when compared to control (C) values; [#,##]represent significant protective effects of MnTBAP ($p<0.05$ and $p<0.01$, respectively).

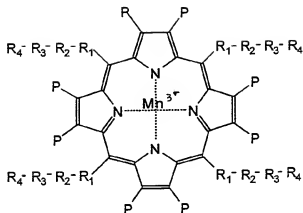Figures 42A-D show synthetic reaction schemes.

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DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to methods of
protecting against the deleterious effects of oxidants,
particularly, superoxide radicals, hydrogen peroxide and
5   peroxynitrite, and to methods of preventing and treating
disease states that involve or result from oxidant
stress. The invention also relates methods of
modulating biological processes involving oxidants,
including superoxide radicals, hydrogen peroxide, nitric
10 oxide and peroxynitrite. The invention further relates
to compounds and compositions, including low molecular
weight antioxidants (eg mimetics of scavengers of
reactive oxygen species, including mimetics of SODs,
catalases and peroxidases) and formulations thereof,
15 suitable for use in such methods.

Mimetics of scavengers of reactive oxygen species
appropriate for use in the present methods include
manganic derivatives of methine substituted porphines,
or pharmaceutically acceptable salts thereof. The
20 methine substituents can be selected so as to facilitate
electron exchange between the mimetic metal (eg Mn) and
the oxygen radical. Substituents that withdraw
electrons from the ring assist in delocalizing the
charge of the metal and thereby enhance catalytic
25 activity. Accordingly, the substituents can be selected
so as to modulate the redox potential of the porphine.
Substituents can also be selected so as to render the
porphyrin resistant to degradation by hemeoxygenase.
Hemeoxygenase, a key enzyme in normal porphyrin

25

degradation and an enzyme that plays a role in the
regulation of inflammation, attacks at the methine
bridge carbons. By designing compounds not susceptible
to attack (eg by introducing substituents at the methine
5   bridge carbons), the half life of the porphyrin can be
increased. Such compounds have the further advantage
that they do not interfere with normal porphyrin
metabolism. Selection of substituents can also be made
based on a result sought to be achieved. For example,
10   when passage through cell membranes is advantageous in a
given treatment regimen, non-polar substituents can be
selected so as to render the mimetic lipid soluble.
Substituents can also be selected so as to render the
mimetic capable of binding to cell surface or
15   extracellular matrix elements. Such substituents can be
selected so as to target the mimetic on the basis of
charge, shape, structure, etc. Targeting substituents
can be specific, for example, for certain cell surface
receptors (eg mannose receptors found on epithelial
20   cells) or for certain sugars or lectins present on the
cell surface.

       In one embodiment, the mimetics are of the formula:

wherein:

R_1 is a bond, —(H), —⟨⟩, —⟨⟩N⁺, —⟨⟩NO₂,

—⟨⟩SO₃H, —⟨⟩SO₃⁻, —⟨⟩X, or —⟨⟩Y wherein X is a halogen

and Y is an alkyl group and wherein ⟨⟩ indicates

bonding to R_2 at any position and ⟨⟩ indicates

bonding to R_2 and the substituent at any position; and

R_2 is a bond, $-(CY'_2)_n^-$, $-(CY'_2-CY'=CY')_n^-$, $-(CY'_2-CY'_2-CH=CH)_n^-$, $-(CY'=CY')_n^-$, or $-(CY'_2-\overset{O}{\overset{\|}{C}})_n^-$, wherein Y'

is hydrogen or an alkyl group and wherein n is 1 to 8;

R_3 is a bond, hydrogen, $-Y''$, $-OH$, $-NH-$, $-N^+(Y'')_3$, $-COO-$, $-COO^-$, $-SO_3-$, $-SO_3^-$, $-C-PO_3H-$ or $-C-PO_3H^-$, wherein Y'' is an alkyl group, and

R_4 is nothing, hydrogen, a cell surface or extra-cellular matrix targeting moiety or a linker-cell surface or extracellular matrix targeting moiety (wherein a "linker" is a moiety that links the mimetic

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core (porphyrin ring and R_1, R_2, R_3) to the targeting moiety).

In a more specific embodiment,

R_1 is a bond, , , N^+, NO_2,

SO_3H, SO_3^-, X, or Y wherein X is Cl or Br and Y is a C_{1-4} alkyl group;

R_2 is a bond, $-(CY'_2)_n^-$, $-(CY'_2-CY'=CY')_n^-$, $-(CY'_2-CY'_2-CH=CH)_n^-$, $-(CY'=CY')_n^-$, or $-(CY'_2-\overset{O}{\overset{\|}{C}})_n^-$, wherein Y' is hydrogen or a C_{1-4} alkyl group and wherein n is 1 to 4;

R_3 is a bond, hydrogen, Y'', -OH, -NH-, $-N^+(Y'')_3$, -COO-, $-COO^-$, $-SO_3-$, $-SO_3^-$, $-C-PO_3H$- or $-C-PO_3H^-$, wherein Y'' is a C_{1-4} alkyl group, and

R_4 is nothing, hydrogen, a cell surface or extracellular matrix targeting moiety or a linker-cell surface or extracellular matrix targeting moiety.

In a further specific embodiment,

R_1 is a bond, , , $N^\pm$, NO_2,

SO_3H, SO_3^-, X, or Y wherein X is Cl or Br and Y is methyl or ethyl, and

R_2 is a bond, $-(CY'_2)_n^-$, $-(CY'_2-CY'=CY')_n^-$, $-(CY'_2-CY'_2-CH=CH)_n^-$, $-(CY'=CY')_n^-$, or $-(CY'_2-\overset{O}{\overset{\|}{C}})_n^-$, wherein Y' is hydrogen or methyl or ethyl and wherein n is 1 or 2; and

R_3 is a bond, hydrogen, methyl, ethyl, -OH, -NH-, $-N^+(CH_3)_3$, $-N^+(CH_2CH_3)_3$, -COO-, $-COO^-$, $-SO_3-$, $-SO_3^-$, $-C-PO_3H$- or $-C-PO_3H^-$, and

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R_4 is nothing, hydrogen, a cell surface or extra-cellular matrix targeting moiety, or a linker-cell surface or extracellular matrix targeting moiety.

In another specific embodiment,

R_1 is a bond, ⬡ , –⬡$N^{\pm}$, ⬡NO_2 , ⬡SO_3H ,

–⬡SO_3^- , or –⬡Y wherein Y is alkyl, preferably, C_{1-4} alkyl, more preferably methyl or ethyl,

R_2 is a bond, $-(CY'_2)_n^-$, $-(CY'=CY')_n^-$, or $-(CY'_2-\overset{O}{\underset{\parallel}{C}})_n^-$, wherein Y' is hydrogen or alkyl (preferably C_{1-4} alkyl, more preferably methyl or ethyl) and wherein n is 1 to 4 (preferably 1 or 2); and

R_3 is a bond, hydrogen, C_{1-4} alkyl (preferably methyl or ethyl), -OH, -NH-, $-N^+(CH_3)_3$, $-N^+(CH_2CH_3)_3$, -COO-, $-COO^-$, $-SO_3-$, $-SO_3^-$, $-C-PO_3H-$ or $-C-PO_3H^-$; and

R_4 is nothing, hydrogen, a cell surface or extra-cellular matrix targeting moiety or a linker-cell surface or extracellular matrix targeting moiety.

In yet another specific embodiment,

R_1 is a bond, ⬡ , –⬡$N^{\pm}$, ⬡NO_2 , ⬡SO_3H , or

–⬡SO_3^-

R_2 is a bond, $-(CY'_2)_n^-$ or $-(CY'=CY')_n^-$, wherein Y' is hydrogen or alkyl (preferably C_{1-4} alkyl, more preferably methyl or ethyl) and wherein n is 1 to 4 (preferably 1 or 2);

R_3 is a bond, hydrogen, C_{1-4} alkyl (preferably methyl or ethyl), -OH, -NH-, $-N^+(CH_3)_3$, $-N^+(CH_2CH_3)_3$, -COO-, $-COO^-$, $-SO_3-$, $-SO_3^-$, $-C-PO_3H-$ or $-C-PO_3H^-$; and

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R_4 is nothing, hydrogen, a cell surface or extra-
cellular matrix targeting moiety or a linker-cell
surface or extracellular matrix targeting moiety.

In addition to the substituents described above,
one or more of the pyrrole rings of the porphyrin can be
substituted (at any or all of carbons 2, 4, 7, 8, 12,
13, 17 or 18) with an electron withdrawing group
(designated P), for example, each P being, indepen-
dently, a NO_2 group, a halogen (eg Cl, Br or F), a
nitrile, a vinyl group, or a formyl group. Such
substituents alter the redox potential of the porphyrin
and thus enhance its ability to scavenge oxygen
radicals. Each P can, independently, also be hydrogen.
Specific mimetics suitable for use in the present
methods include Mn(III) tetrakis (1-methy-4-
pyridyl)porphyrin (MnTMPyP), Mn(III) tetrakis (4-
trimethyl-aminophenyl)porphyrin (MnTMAP) and Mn(III)
tetrakis (4-benzoic acid)porphyrin (MnTBAP), with or
without a cell surface or extracellular matrix targeting
moiety or a linker-cell surface or extracellular matrix
targeting moiety at the R_4 position.

Although the foregoing mimetics are described as
manganese chelates, metals other than manganese, such as
iron (III) and copper (II), can also be used. The
present invention also relates to the metal-free
nitrogen-containing macro cyclic ligand. It will be
appreciated that the metal selected may have various
valence states, for example, manganese II, III or V can
be used. The change in charge will be dependent upon
the acceptance or release of electrons.

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In addition to the foregoing mimetics, the invention also includes compounds (and targeted forms thereof as described above, including linker-cell surface or extracellular matrix targeted forms) of the formula:
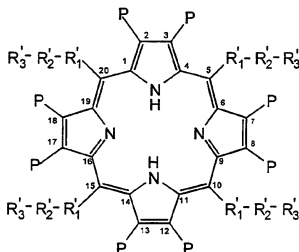
or a pharmaceutically acceptable salt thereof, or metal complex thereof wherein the metal is, for example, manganese, copper or iron,

wherein:

each R_1' is independently a bond, ![structure]—CO₂Y" , ![structure]—OH ,
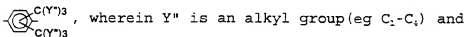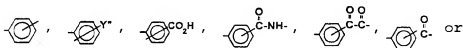
![structure] , ![structure]—Y" , ![structure]—CO₂H , ![structure]—C-NH- , ![structure]—C-C- , ![structure]—C- or

![structure]—C(Y")₃ , wherein Y" is an alkyl group (eg C_1-C_4) and

wherein ![structure] indicates bonding to R_2' at any position

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
and indicates bonding to R_2' and the R_1' phenyl

substituent at any position;

each R_2' is independently a bond, or $-(CH_2)_n-$
wherein n is 1-4,

5   each R_3' is independently -Y", -Y'", -H, -OH, -OY",
-NO$_2$, -CN, -NH$_2$, -COOH, -COY", -COO$^-$, or a heterocyclic
group, wherein Y" is as defined above and Y'" is a
primary, secondary, tertiary or quaternary amine
(preferably, an alkyl amine wherein the alkyl groups
10   are, for example, C_1-C_5 alkyls)

wherein when R_1' is , R_3' is not COOH, COY"

or COO$^-$,

wherein when R_1' is , or , R_3'

is not -NO$_2$.

15   In certain embodiments of the invention, $-R_1'-R_2'-$

R_3', collectively, are not -H, , , ,

, or , for example, when Y" is

a methyl.

20   As indicated above, R_3' can represent a hetero-
cyclic moiety. Possible heterocyclics include substi-
tuted or unsubstituted tetrazoles, furans, thiophenes,
indoles, imadazoles, pyridines, oxadiazoles and

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quinolines. Possible substituents on such moieties
include halogen (eg Br or Cl), $-NO_2$, C_{1-4} alkyl, and C_{1-4}
alkyl alcohol groups.

5   As above, one or more of the pyrrole rings of the
porphyrin can be substituted (at any or all of carbons
2, 4, 7, 8, 12, 13, 17 or 18) with an electron with-
drawing group (designated P), for example, each P being,
independently, a NO_2 group, a halogen (eg Cl, Br or F),
a nitrile, a vinyl group, or a formyl group. P can also
10   be hydrogen.

           Where rotational isomers are possible, all such
isomers of the herein described mimetics (oxidant
scavengers) are within the scope of the invention.

           In addition to the compounds described above, the
15   present invention includes within its scope porphine
mimetics (and targeted forms thereof) bearing any of the
above substituents in any of the possible combinations.
For example R_1 can be selected from the group described
above with respect to R_1 or R_1', R_2 can be selected from
20   the group described above with respect to R_2 and R_2', and
R_3 can be selected from the group described above with
respect to R_3 or R_3'.

           Targeted forms of the mimetics can be produced by
coupling the porphine directly or via a linker to a cell
25   surface or extracellular matrix targeting moiety, for
example, as indicated above in the definition of R_4.
The targeted mimetics can be used, for example, to mimic
EC-SOD. Since the sequence of human EC-SOD is known
(Hjalmarsson et al, Proc. Natl. Acad. Sci. USA 84:6340
30   (1987)), the C-terminal oligopeptide can be prepared and

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attached to the "mimetic core" (eg, a Mn(III)-porphyrin)
via, for example, a free amine or carboxy group with a
1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC)
coupling reaction (Yamada et al, Biochemistry 20:4836
5   (1981); Davis and Preston, Anal. Biochem. 116:402
(1981)). This simple coupling procedure makes it
possible to attach a wide variety of different binding
domains in order to target the mimetics of the
invention. Heparin binding affinity of a mimetic can be
10   assessed by passing the mimetic over a heparin-sepharose
CL-6B column (Karlsson et al, Biochem. J. 256:29
(1988)).

Candidate targeting moieties suitable for attaching
to the mimetics of the invention to confer GAG binding
15   properties include the following:
i)   the A+ helix of protein C inhibitor
(Boissinot et al, Biochem. Biophys. Res. Commun. 190:250
(1993)) - NH_2 - His Arg His His Pro Arg Glu Met Lys Lys
Arg Val Glu Asp Leu - COOH;
20   ii)   the C-terminal end of human EC-SOD (heparin
binding domain) (Karlsson et al, Biochem. J. 255:223
(1988)) - NH_2 - Arg Glu His Ser Glu Arg Lys Lys Arg Arg
Arg Glu Ser Glu Cys Lys Ala Ala - COOH;
iii)   variants of the C-terminal end of human
25   EC-SOD having heparin binding affinity (Sandström et al,
J. Biol. Chem. 267:18205 (1992)) -
a. NH_2 - Arg Glu His Ser Glu Arg Lys Lys Arg Arg Arg
Glu - COOH;
b. NH_2 - Arg Glu His Ser Glu Arg Lys Lys Arg Arg Arg
30   Ala - COOH;

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c.   NH$_2$ - Arg Glu His Ser Glu Arg Lys Lys Arg Arg Arg
Ala Ser Glu Cys Lys Ala Ala - COOH;

d.   NH$_2$ - Arg Glu His Ser Glu Arg Lys Lys Arg Arg Arg
Glu Ser Glu Ala Lys Ala Ala - COOH;

e.   NH$_2$ - Arg Glu His Ser Glu Arg Lys Lys Arg Arg Arg
Glu Ser Glu Cys Ala Ala Ala - COOH;

f.   NH$_2$ - Arg Glu His Ser Glu Arg Lys Lys Arg Arg Arg
Ala Ser Ala Cys Lys Ala Ala - COOH;

g.   NH$_2$ - Arg Glu His Ser Glu Arg Lys Lys Arg Arg Arg
Ala Ser Glu Cys Ala Ala Ala - COOH;

h.   NH$_2$ - Arg Glu His Ser Glu Arg Lys Lys Gly Arg Arg
Ala Ser Glu Cys Ala Ala Ala - COOH;

iv)   any combination of repeating positively
changed amino acids (ie poly (Arg)$_n$, poly (Lys)$_n$, poly
(Arg)$_n$ (Lys)$_n$ or poly (Arg Lys)$_n$ or poly (Lys Arg)$_n$,
poly (Lys Lys Arg Arg)$_n$, wherein n is, preferably, in
the range of 1 to 12, more preferably, 1 to 8, and, most
preferably, 1 to 6, with or without a C-terminal Glu or
Ala (Sandström et al, J. Biol. Chem. 267:18205 (1992));
and

v)   polyethyleneimine, e.g. (NH-CH$_2$-CH$_2$-NH)nH,
wherein n is 1 to 6.

In addition to the foregoing, targeting moieties
also include heparin binding proteins generally and
sugars, including mannose and oligosaccharides.

Appropriate linkers include -$\overset{\overset{O}{\|}}{C}$-NH- or -NH-$\overset{\overset{O}{\|}}{C}$-,
-SO$_2$-NH-, -PO$_3$-NH-, or -PO$_3$-$\overset{\overset{O}{\|}}{C}$-.

Specific examples of suitable mimetics of the
invention are set forth below:

$$R = \text{—} \langle \text{phenyl} \rangle \text{—} N^+(CH_3)_3$$

$$R = \text{—} \langle \text{pyridyl} \rangle N^+\text{-}CH_3$$

$$R = \text{—} \langle \text{phenyl} \rangle \text{—COOH}$$

$$R = \text{—} \langle \text{phenyl} \rangle \text{—}NH_2$$

$$R = \text{—} \langle \text{phenyl} \rangle \text{—CONHR'}$$

$$R = \text{—} \langle \text{phenyl} \rangle \text{—NHCOR'}$$

R' = NH$_2$-ArgGluHisSerGluArgLysLysArgArgArg
GluSerGluCysLysAlaAla-COOH
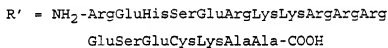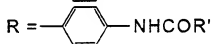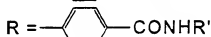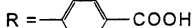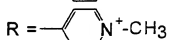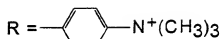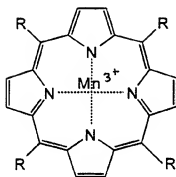
5   5,10,15,20-Tetra Kis [R-group]
           manganese (III) porphyrins

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SUBSTITUTE SHEET (RULE 26)

Mn-OB-TMPyP

Mn-OC-TMPyP

Mn-OF-TMPyP

Mn-ON-TMPyP

SUBSTITUTE SHEET (RULE 26)

Mn-OB-TBAP

Mn-OC-TBAP

Mn-OF-TBAP

Mn-ON-TBAP

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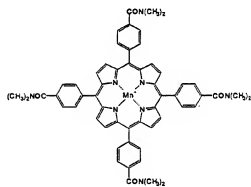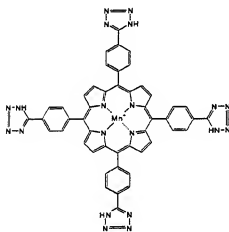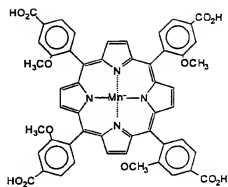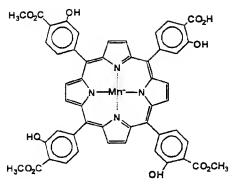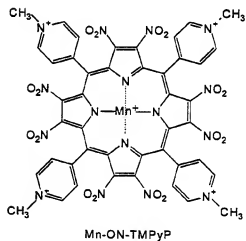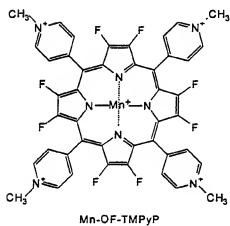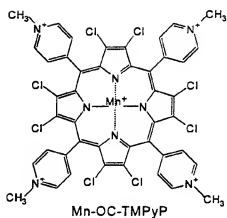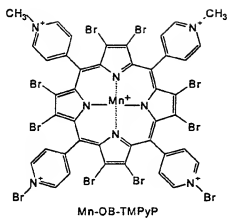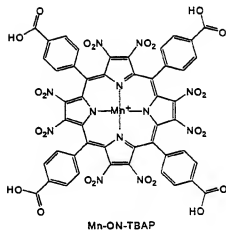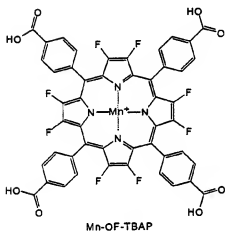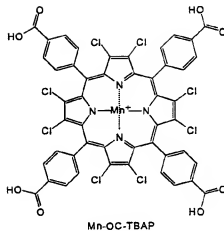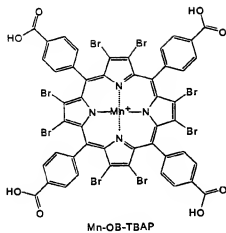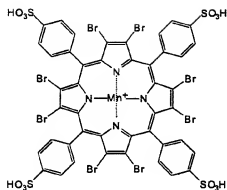
Mn-OB-TPSP
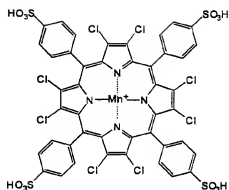
Mn-OC-TPSP
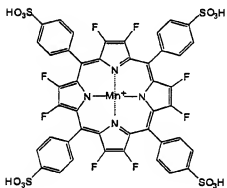
Mn-OF-TPSP

Mn-ON-TPSP

40
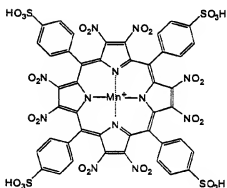
Mimetics suitable for use in the present methods
can be selected by assaying for SOD, catalase and/or
peroxidase activity and stability. The selective,
reversible and SOD-sensitive inactivation of aconitase
5   by known O_2^- generators can be used as a marker of O_2^-
generation. Thus, suitable mimetics can be selected by
assaying for the ability to protect aconitase activity.
SOD activity can be monitored in the presence and
absence of EDTA using the method of McCord and Fridovich
10   (J. Biol. Chem. 244:6049 (1969)). The efficacy of a
mimetic can be determined by measuring the effect of the
mimetic on the growth of a SOD null *E. coli* strain
versus a wild type strain. Specifically, wild type *E.
coli* (AB1157) and SOD null *E. coli.* (JI132) can be grown
15   in M9 medium containing 0.2% casamino acids and 0.2%
glucose at pH 7.0 and 37°C; growth can be monitored in
terms of turbidity followed at 700 nm
spectrophotometrically. Active mimetics can be tested
for toxicity in mammalian cell culture by measuring
20   lactate dehydrogenase (LDH) release. Specifically, rat
L2 cells (a lung Type II like cell; (Kaighn and Douglas,
J. Cell Biol. 59:160a (1973))can be grown in Ham's F-12
medium with 10% fetal calf serum supplement at pH 7.4
and 37°C; cells can be seeded at equal densities in 24
25   well culture dishes and grown to approximately 90%
confluence; SOD mimetics can be added to the cells at
log doses (eg micromolar doses in minimal essential
medium (MEM)) and incubated for 24 hours. Toxicity can
be assessed by morphology and by measuring the release
30   of the cytosolic injury marker, LDH (eg on a

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thermokinetic plate reader), as described by Vassault
(In: Methods of Enzymatic Analysis, Bergmeyer (ed)
pp. 118-26 (1983); oxidation of NADH is measured at
340 nm). Efficacy of active mimetics can be assessed by
5   determining their ability to protect mammalian cells
against methylviologen (paraquat)-induced toxicity.
Specifically, rat L2 cells grown as described above and
seeded into 24 well dishes can be pre-incubated with
various concentrations of the SOD mimetic and then
10   incubated with a concentration of methylviologen
previously shown to produce an LC_{75} in control L2 cells.
Efficacy of the mimetic can be correlated with a
decrease in the methylviologen-induced LDH release
(St. Clair et al, FEBS Lett. 293:199 (1991)). The
15   efficacy of SOD mimetics can be tested *in vivo* with
mouse and/or rat models using both aerosol
administration and parenteral injection. For example,
male Balb/c mice can be randomized into 4 groups of 8
mice each to form a standard 2X2 contingency statistical
20   model. Animals can be treated with either paraquat
(40 mg/kg, ip) or saline and treated with SOD mimetic or
vehicle control. Lung injury can be assessed 48 hours
after paraquat treatment by analysis of bronchoalveolar
lavage fluid (BALF) damage parameters (LDH, protein and
25   % PMN) as previously described (Hampson et al, Tox.
Appl. Pharm. 98:206 (1989); Day et al, J. Pharm. Methods
24:1 (1990)). Lungs from 2 mice of each group can be
instillation-fixed with 4% paraformaldehyde and
processed for histopathology at the light microscopic
30   level.

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Catalase activity can be monitored by measuring
absorbance at 240nm in the presence of hydrogen peroxide
(see Beers and Sizer, J. Biol. Chem. 195:133 (1952)) or
by measuring oxygen evolution with a Clark oxygen
5   electrode (Del Rio et al, Anal. Biochem. 80:409 (1977)).
Peroxidase activity can be measured
spectrophotometrically as previously described by Putter
and Becker: Peroxidases. In: Methods of Enzymatic
Analysis, H.U. Bergmeyer (ed.), Verlag Chemie, Weinheim,
10   pp. 286-292 (1983. Aconitase activity can be measured
as described in Example XI below.

Table IX below provides a summary of the activities
of various oxidant scavengers of the invention. The
footnote to that Table provides details of the assays
15   used.

Synthesis of mimetics suitable for use in the
present method can be effected using art-recognized
protocols. Example XIV includes a detailed description
of the synthesis of four specific mimetics. In the case
20   of Mn(III)-porphyrin mimetics, porphyrin rings with
various methine bridge carbon side groups can be
purchased commercially and the Mn(III) metal ion
inserted into the porphyrin ring by methods including
the following: (1) admixture of Mn(II) acetate with
25   porphyrin in the presence of oxygen, under which
condition selective stabilization of Mn(III) by the
porphyrin causes autooxidation of Mn(II);
(2) preparation of $Mn(III)(OH)_3$ by a modification of the
Winkler method (Sastry et al, Anal. Chem. 41:857 (1969))
30   followed by reaction with the porphyrin; (3) stirring

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MnO_2 with the porphyrin in the presence of NH_2OH, which
serves to reduce the Mn(IV) to Mn(III), which is then
trapped by the porphyrin; or (4) a method modified from
Pasternack et al (Biochemistry 22:2406 (1983)) which

5   refluxes excess $MnCl_3$ with the porphyrin. Mn(III)-
porphyrin complexes can be precipitated from solution
with sodium perchlorate, washed and residue perchlorate
removed by strong anionic exchange resin. Formation of
the Mn(III)-porphyrin can be followed

10   spectrophotometrically by monitoring a characteristic
Sorét band at 468 nm. Synthesis of compounds of the
invention bearing electron-withdrawing groups at one or
more pyrrole carbons can be carried out as described by
Richards et al, Inorg. Chem. 35:1940 (1996).

15   Purification of the mimetics can be effected using art-
recognized techniques such as recrystallization,
chromatography, etc. Coupling of a binding domain to
the "mimetic core" can be carried out as described
above.

20   One embodiment of the present invention results, at
least in part, from the realization that EC-SOD
specifically regulates NO· function. In addition, the
invention is based on the realization that EC-SOD is
synthesized by epithelial cells and is primarily

25   localized in the interstitium, on matrix elements and
collagen and around smooth muscle cells (particularly
lung airways and vasculature). NO· is an intercellular
signal and, as such, NO· must traverse the extracellular
matrix to exert its effects. NO·, however, is highly

30   sensitive to inactivation mediated by O_2^- present in the

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extracellular spaces. EC-SOD is thus an enzyme ideally
suited to increase the bioavailability of NO· by
preventing its degradation by O_2^-.

One embodiment of the present invention relates to
5   a method of regulating extracellular NO· levels using
polypeptides having EC-SOD activity. As indicated
above, the invention also relates to mimetics of EC-SOD
that can be targeted to strategic locations and to the
use of such mimetics in manipulating extracellular
10   levels of NO·. The invention, however, is not limited
to NO· manipulation as the sole mechanism of action of
the compounds, mimetics, etc, of the invention. Rather,
the invention relates to oxygen radical, hydrogen
peroxide and peroxynitrite scavenging generally.

15   The present invention relates, in a further
specific embodiment, to a method of inhibiting
production of superoxide radicals. In this embodiment,
the mimetics of the invention are used to inhibit
oxidases, such as xanthine oxidase, that are responsible
20   for production of superoxide radicals (see Example VII).
The ability of a mimetic to protect mammalian cells from
xanthine/xanthine oxidase-induced injury can be
assessed, for example, by growing rat L2 cells in 24-
well dishes. Cells can be pre-incubated with various
25   concentrations of a mimetic and then xanthine oxidase
(XO) can be added to the culture along with xanthine
(X). The appropriate amount of XO/X used in the study
can be pre-determined for each cell line by performing a
dose-response curve for injury. X/XO can be used in an
30   amount that produces approximately an LC_{75} in the

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culture. Efficacy of the mimetic can be correlated with
a decrease in XO/X-induced LDH release. The ability of
the mimetics to inhibit the production of such radicals
makes possible the use the mimetics as therapeutics for
5   the treatment of gout and reperfusion injuries.

The mimetics of the invention can be used as
catalytic scavengers of reactive oxygen species to
protect against ischemia reperfusion injuries associated
with myocardial infarction, stroke, acute head trauma,
10   organ reperfusion following transplantation, bowel
ischemia, pulmonary infarction, surgical occlusion of
blood flow, and soft tissue injury. The mimetics can
further be used to protect against skeletal muscle
reperfusion injuries. The mimetics can also be used to
15   protect against damage to the eye due to sunlight (and
to the skin) as well as glaucoma, and macular
degeneration of the eye. Diseases of the bone are also
amenable to treatment with the mimetics. Further,
connective tissue disorders associated with defects in
20   collagen synthesis or degradation can be expected to be
susceptible to treatment with the present mimetics.

In addition to scavenging superoxide, the ability
of the mimetics of the invention to scavenge hydrogen
peroxide would protect from the possible formation of
25   the highly reactive hydroxyl radical by interfering with
Fenton chemistry (Aruoma and Halliwell, Biochem. J.
241:273 (1987); Mello Filho et al, Biochem. J. 218:273
(1984); Rush and Bielski, J. Phys. Chem. 89:5062 (1985).
These metalloporphyrins have been shown to scavenge
30   peroxynitrite as demonstrated indirectly by inhibition

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of the oxidation of dihydrorhodamine 123 to rhodamine
123 (see Example XIII) and directly by accelerating
peroxynitrite degradation by stop flow analysis.

In addition to the above, the mimetics of the
5   invention can be used as catalytic scavengers of
reactive oxygen species to increase the very limited
storage viability of transplanted hearts, kidneys, skin
and other organs and tissues. The invention also
provides methods of inhibiting damage due to
10   autoxidation of substances resulting in the formation of
O_2 including food products, pharmaceuticals, stored
blood, etc. To effect this end, the mimetics of the
invention are added to food products, pharmaceuticals,
stored blood and the like, in an amount sufficient to
15   inhibit or prevent oxidation damage and thereby to
inhibit or prevent the degradation associated with the
autoxidation reactions. (For other uses of the mimetics
of the invention, see USP 5,227,405). The amount of
mimetic to be used in a particular treatment or to be
20   associated with a particular substance can be determined
by one skilled in the art.

The availability of the mimetics of the invention
also makes possible studies of processes mediated by O_2,
hydrogen peroxide, nitric oxide and peroxynitrite.

25   In addition to the above, the present invention
relates to diagnostic protocols made possible by the
availability of the EC-SOD gene sequence (see Example V
and Figure 24). A defect in the EC-SOD gene is more
likely to occur than a defect in nitric oxide synthase
30   due to the nature and number of physiological functions

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served by NO·. Detection of an EC-SOD gene defect would
signal the need for measures to be undertaken to elevate
levels of functional EC-SOD, or related superoxide
scavenging compounds, at strategic locations to correct
5   NO· imbalances and thus disorders involving oxidative
stress.

To effect modulation of the efficacy of extracellular
NO·, eg, in relaxing smooth muscle, molecules (agents)
having EC-SOD activity are administered under conditions
10   such that levels of extracellular O_2^- are altered.
Molecules suitable for use in this method include
forms of SOD that bind heparin sulfate or other
glycosaminoglycans (GAG), for example, by virtue of the
fact that they contain positively charged amino acids
15   near their carboxy terminal end. Proteinaceous agents
suitable for use in the present method include forms of
EC-SOD C described in WO 91/04315, as well as additional
polypeptides defined and described therein as having
comparable or enhanced binding to heparin as compared to
20   recombinant EC-SOD C (eg, polypeptides G1 and SA216;
note also polypeptide SA219 which has the same heparin
binding as recombinant EC-SOD C. Further proteinaceous
agents suitable for use in the present method include
chimeric proteins with targeted binding and SOD
25   activity, for example, Cu/Zn SOD linked to an EC-SOD
binding sequence (see also Boissinot et al, Biochem.
Biophys. Res. Commun. 190:250 (1993)).

Proteinaceous molecules suitable for use in the
present method can be synthesized chemically or
30   recombinantly using art-recognized protocols (see

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WO 91/04315). Non-glycosylated recombinant peptides can
be produced, for example, using host cells (ie, *E. coli*
cells) that are incapable of effecting glycosylation, or
using DNA sequences encoding functional proteins lacking
5   glycosylation sites.

In addition to polypeptides, molecules suitable for
use in the present method include mimetics of EC-SOD (eg
targeted mimetics), including those described above.
The general requirements of such mimetics are that they:
10   (a) be stable enough to retain the ligated metal (eg Cu
or Mn) in the presence of the multiple chelating agents
present in living systems, (b) be active enough that
reasonable doses can serve to significantly augment the
total SOD activity in the extracellular spaces, (c) be
15   able to adhere to the surfaces of cells or extracellular
matrix elements (eg collagen) when protection against
extracellular sources of O_2^- is needed, and d) be of low
toxicity. Examples of suitable mimetics include
nitrogen-containing macrocyclic ligands effective as
20   catalysts for dismutating superoxide, including Mn(III)
complexes of porphyrins with bulky cationic substituents
on the methine bridge carbons, such as those described
above (eg MnTMAP and MnTMPyP). Such complexes are very
active and are stable enough to retain full activity in
25   the presence of excess EDTA or in the presence of tissue
extracts.

The polypeptides and mimetics described above can
be formulated into pharmaceutical compositions suitable
for use in the present methods. Such compositions
30   include the active agent (polypeptide or mimetic)

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together with a pharmaceutically acceptable carrier,
excipient or diluent. The composition can be present in
dosage unit form for example, tablets, capsules or
suppositories. The composition can also be in the form
of a sterile solution suitable for injection or
nebulization. Compositions can also be in a form
suitable for opthalmic use. The invention also includes
compositions formulated for topical administration, such
compositions taking the form, for example, of a lotion,
cream, gel or ointment. The concentration of active
agent to be included in the composition can be selected
based on the nature of the agent, the dosage regimen and
the result sought.

The dosage of the composition of the invention to
be administered can be determined without undue
experimentation and will be dependent upon various
factors including the nature of the active agent, the
route of administration, the patient, and the result
sought to be achieved. A suitable dosage of protein
administered IV can be expected to be in the range of
about 10-1000 mg/day. For topical treatment, it is
expected that lower doses will be required (see
WO 91/04315); for aerosol administration, it is expected
that doses will be in the range of 1 to 10 mg/kg.
Suitable doses of mimetics will vary, for example, with
the mimetic and with the result sought. The results of
Faulkner et al (J. Biol. Chem. 269:23471 (1994))
indicate that the *in vivo* oxidoreductase activity of the
mimetics is such that a pharmaceutically effective dose
will be low enough to avoid problems of toxicity. Doses

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that can be used include those in the range of 1 to 50
mg/kg.

In addition to compositions of the types described
above, the present invention also includes compositions
5   suitable for use in gene therapy types of protocols.
For example, the invention includes DNA sequences
encoding proteins having EC-SOD activity and formulated
so as to be incorporated into cells (eg, lung cells)
upon contact therewith (eg, via inhalation). The
10   sequence can be present in a vector, eg, a viral vector,
and/or the sequence can be present in a delivery
vehicle, such as a liposome. The amounts of such
compositions to be administered can be readily
determined by one skilled in the art.

15   Further examples of diseases or disorders
appropriate for treatment using the compounds and
compositions of the present invention include diseases
of the central nervous system (including AIDS dementia,
stroke, amyotrophic lateral sclerosis (ALS), Parkinson's
20   disease and Huntington's disease) and diseases of the
musculature (including diaphramic diseases (eg
respiratory fatigue in emphysema, bronchitis and cystic
fibrosis), cardiac fatigue of congestive heart failure,
muscle weakness syndromes associated with myopathies,
25   ALS and multiple sclerosis). Many neurologic disorders
(including stroke, Huntington's disease, Parkinson's
disease, ALS, Alzheimer's and AIDS dementia) are
associated with an over stimulation of the major subtype
of glutamate receptor, the NMDA (or N-methyl-D-
30   aspartate) subtype. On stimulation of the NMDA

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receptor, excessive neuronal calcium concentrations
contribute to a series of membrane and cytoplasmic
events leading to production of oxygen free radicals and
nitric oxide (NO·). Interactions between oxygen free
5   radicals and NO· have been shown to contribute to
neuronal cell death. Well-established neuronal cortical
culture models of NMDA-toxicity have been developed and
used as the basis for drug development. In these same
systems, the mimetics of the invention inhibit NMDA
10   induced injury. The results presented in Example XI
demonstrate that the formation of O_2^- radicals is an
obligate step in the intracellular events culminating in
excitotoxic death of cortical neurons and further
demonstrate that the mimetics of the invention can be
15   used to scavenge O_2^- radicals and thereby serve as
protectants against excitotoxic injury. Compound 10303
(see Table IX) decreases NMDA- and kainate-induced
excitotoxicity in rat cortical cells in a dose dependent
manner with 100% protection against NMDA-induced
20   excitotoxicity (50 μM) being achieved at 25 μM compound
10303, 100 μM compound 10303 in the case of kainate-
induced excitotoxicity (300 μM).

The present invention also relates to methods of
treating arthritis, systemic hypertension,
25   atherosclerosis, edema, septic shock, pulmonary
hypertension, including primary pulmonary hypertension,
impotence, infertility, endometriosis, premature uterine
contractions, microbial infections, gout and in the
treatment of Type II diabetes mellitus. The scavengers
30   of the invention can be used to ameliorate the toxic

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effects associated with endotoxin, for example, by
preserving vascular tone and preventing multi-organ
system damage.

5   The invention further relates to methods of
treating memory disorders. While not wishing to be
bound by theory, it is believed that nitric oxide is a
neurotransmitter involved in long-term memory
potentiation. Using an EC-SOD knocked-out mouse model,
it can be shown that learning impairment correlates with

10   reduced superoxide scavenging in extracellular spaces of
the brain (see Example XII). Reduced scavenging results
in higher extracellular O_2^- levels. O_2^- is believed to
react with nitric oxide thereby preventing or inhibiting
nitric oxide-medicated neurotransmission and thus long-

15   term memory potentiation. The mimetics of the invention
can be used to treat dementias and memory/learning
disorders.

The Therapeutic regimens, including mode of
administration, appropriate for effecting treatment of

20   the conditions described above can be readily determined
by one skilled in the art.

Inflammations, particularly inflammations of the
lung, are amenable to treatment using the present
invention (note particularly the inflammatory based

25   disorders of asthma, ARDS including oxygen toxicity,
pneumonia (especially AIDS-related pneumonia), cystic
fibrosis, chronic sinusitis and autoimmune diseases
(such as rheumatoid arthritis)). EC-SOD is localized in
the interstitial spaces surrounding airways and

30   vasculature smooth muscle cells. EC-SOD and O_2^- mediate

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the antiinflammatory - proinflammatory balance in the
alveolar septum. NO· released by alveolar septal cells
acts to suppress inflammation unless it reacts with O_2^-
to form $ONOO^-$. By scavenging O_2^-, EC-SOD tips the
balance in the alveolar septum against inflammation.
Significant amounts of $ONOO^-$ will form only when EC-SOD
is deficient or when there is greatly increased O_2^-
release. EC-SOD mimetics, such as those described
herein, can be used to protect against destruction
caused by hyperoxia. Appropriate therapeutic regimens
can be readily established by one skilled in the art.

     As indicated above, it is expected that defects in
the EC-SOD gene, that are manifest in the protein
itself, may in fact be the cause of pathologic problems
related to NO· function, rather than defects in nitric
oxide synthase. Thus, in a further embodiment, the
present invention relates to diagnostic protocols
suitable for use in identifying EC-SOD gene defects.
This aspect of the invention is based on the
availability of the EC-SOD gene sequence. The present
invention includes within its scope the gene sequence
presented in Figure 24 as well as portions of non-coding
regions of that sequence of at least 15 bases,
preferably, at least 50 bases, more preferably, at least
100 bases and most preferably, at least 500 bases. Such
portions, and the complements thereof, which are also
within the scope of the invention, can be used as probes
or primers in protocols including those described in
Example VI.

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Screening of subjects for defects in the EC-SOD gene can be done using the approach used to identify mutations on the ß-adrenergic receptor gene (Reihaus et al, Am. J. Respir. Cell. Mol. Biol. 8:334 (1993)). That

5   approach is similar to the one used by Rosen et al (Nature 262:59 (1993)) (see Example VI).

The following are predicted sites of important gene mutations:

Positions 1-558: This represents a 5' flanking

10   region which contains transcriptional regulatory elements. Mutations here can be expected to lead to deficient levels of EC-SOD or defective enhancement or reduction in EC-SOD levels under conditions which require manipulating the EC-SOD concentration. The

15   following regions have been identified as putative regulatory regions. Mutations in these regions can be expected to result in deficient levels of EC-SOD:

89 - 95   metal regulatory response element
121 - 126 cyclic AMP responsive element
20   370 - 375 glucocorticoid response element
238 - 244 skeletal muscle trans-activating factor
                    response element
251 - 256 cis responsive element in the induction
                    of the c-fos proto-oncogene
25   162 - 168 TPA reponsive element
171 - 179 SV40 enhancer region

Positions 560-570: Mutations here can be expected to lead to an inability to splice out intron 1. This

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would result in no EC-SOD production (or much reduced)
due to initiation of translation at multiple cryptic ATG
sites located in intron 1 which are upstream of the
EC-SOD ATG start codon. For example, base pair 653,
5   656, 720, 743, 748, etc, would potentially initiate
translation.

Positions 564-1135: Intron 1 contains DNA sequence
unique to EC-SOD. In addition, there are potential
transcription regulatory regions within this DNA stretch
10   which are listed below; mutations in intron 1 would lead
to deficient levels of EC-SOD or defective enhancement
or reduction in EC-SOD levels under conditions that
require manipulating the EC-SOD concentration:

1085 - 1095   Xenobiotic responsive region
15   650 - 661   Antioxidant responsive element

Positions 71-95: Mutations here can be expected to
lead to an inability to splice out intron 1. This would
result in no EC-SOD production (or much reduced) due to
initiation of translation at multiple cryptic ATG sites
20   located in intron 1 that are upstream of the EC-SOD ATG
start codon. For example, base pair 653, 656, 720, 743,
748, etc, would potentially initiate translation.

Positions 1211-1230: Mutations here can be
expected to lead to an inability to splice out intron 2.
25   This would result in no EC-SOD production (or much
reduced) due to initiation of translation at multiple
cryptic ATG sites located in intron 2 that are upstream

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of the EC-SOD ATG start codon. Examples of upstream ATG
translation start sites can be found at 1339 and 1518.

   Positions 5055-5080: Mutations here would lead to
an inability to splice out intron 2. This would result
5 in no EC-SOD production (or much reduced) due to
initiation of translation at multiple cryptic ATG sites
located in intron 2 which are upstream of the EC-SOD ATG
start codon. Examples of upstream ATG translation start
sites can be found at 1339 and 1518.

10   Positions 5085-5138: Mutations here would (1)
interfere with efficiency of translation of EC-SOD
resulting in deficient levels of the enzyme (2)
interfere with targeting of EC-SOD to the endoplasmic
reticulum which is required for secretion of EC-SOD, (3)
15 interfere with co-translational signal peptide
processing (ie, removal of the signal peptide) that may
lead to deficient levels due to inability to
proteolytically cleave the signal peptide from the
mature protein which in turn would result in the protein
20 being trapped in the endoplasmic reticulum, (4)
interfere with post-translational processing
(specifically glycosylation) which may result in
defective levels due to the synthesis of poorly soluble
protein.

25   Positions 5139-5150: Mutations here may interfere
with the signal peptidase cleavage site resulting in a
mutant EC-SOD which would contain an altered amino
terminus leading to defective EC-SOD function of
deficient levels.

Positions 5403-5405: Mutations here can be
expected to result in loss of glycosylation which may
result in defective levels due to the synthesis of
poorly soluble protein.

Positions 5424-5720: Mutations here can be
expected to result in defective EC-SOD activity. This
region is critical for binding to the substrate and
catalyzing the dismutation of superoxide anion radical.
In addition, this region would also affect any other
activities of this enzyme including the reduction of
other species such as nitric oxide, etc.

Positions 5721-5804: Mutations in this region
would cause defects in binding of EC-SOD to target
tissues such as type I collagen in the extracellular
matrix, and around smooth muscle cells in both vessels
and bronchi. Such mutations here are highly likely to
cause disease.

Positions 6385-6395: Mutations here can be
expected to result in defective polyadenylation that can
lead to deficient levels of EC-SOD due to a decreased
half-life of EC-SOD mRNA species.

The present invention relates not only to the
entire EC-SOD gene sequence, but to portions thereof for
use, for example, as probes or primers in detecting
mutations in regions of the gene including those noted
above. Such portions can be expected to be, for
example, 18-1500 bases in length, preferably 18-22 bases
in length, 50-75 bases in length, 100-400 bases in
length or 500 to 1500 bases in length.

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Certain details of the present invention are
described in greater detail in the non-limiting Examples
that follow.

Example I

5   Preparation and Characterization of Transgenic Mice

Protocols:

i)   Construction of Transgenic Mice

Construction of the human EC-SOD expression vector:
The EC-SOD expression vector (Figure 1) was constructed
10   as follows: The entire human EC-SOD cDNA fragment
(Hjalmarrson et al, Proc. Natl. Acad. Sci. USA 84:6340
(1987); Hendrickson et al, Genomics 8:736 (1990))
flanked by EcoRI restriction sites was converted with
mung bean nuclease to form blunt-ends, ligated to SalI
15   linkers, digested with SalI, and then inserted into the
SalI site of the human ß-actin expression vector
pHßAPr-1. The EcoRI-HindIII fragment of the resultant
plasmid containing the human ß-actin promoter (provided
by Dr. Larry Kedes of the University of Southern
20   California, Los Angeles, California), intron, and EC-SOD
cDNA was isolated. In addition, the HpaI site of SV40
at position 2666 in plasmid pMSG (Pharmacia LKB
Biotechnology, Piscataway, New Jersey) was converted to
a HindIII site by linker ligation and the HindIII-PstI
25   fragment containing the polyadenylation site of the SV40

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early region was isolated. These two DNA fragments were
then ligated to an EcoRI plus PstI digested pKS vector
(Stratogene, La Jolla, California). The EcoRI-XbaI
fragment containing the entire expression construct free
5   of plasmid sequences was isolated and used to establish
transgenic mice. All of the recombinant DNA procedures
were done according to established methods (Sambrook et
al, Molecular Cloning: A Laboratory Manual 3, Cold
Spring Harbor; Cold Spring Harbor Laboratory, 1989).

10   _Development of transgenic mice_: Purified DNA at
2.5 µg/ml in 5 mM Tris-HCl, pH 7.4, 0.1 mM EDTA was
injected into the pronuclei of fertilized eggs isolated
from mice ((C57BL/6 X C3H)F1 X (C57BL/6 X
C3H)F1)((C57BL/6 X C3H)F1 mice were purchased from
15 Charles River). Mouse eggs surviving microinjection
were then implanted into the oviducts of pseudopregnant
foster mothers (CD1) (CD1 mice were purchased from
Charles River) following procedures described by Hogan
et al (Hogan et al, Manipulating the Mouse Embryo, Cold
20 Spring Harbor; Cold Spring Harbor Laboratory 1986, 32).
Mice carrying the transgene were identified by Southern
blot analysis of tail DNA probed with the entire human
EC-SOD cDNA. Transgenic founders were found the first
litter screened. These mice were bred with (C57BL/6 X
25 C3H)FI to produce offspring for further studies. (In
all of the following experiments with the EC-SOD
transgenic mice, the nontransgenic mice refer to
littermates of the transgenic mice that did not contain
the transgene for the human EC-SOD. In experiments in

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which EC-SOD transgenic mice were not used, wild type
(C57BL/6 X C3H)F1 were used.)

Production of homozygous EC-SOD transgenic mice:
Homozygous transgenic mice were produced by an F_1 cross

5   of heterozygous transgenic mice. Tail DNA from F_2 mice
were isolated and treated with RNAase. 10 μg of DNA
from each mouse was cut with PstI and then
electrophoresed through a 1.2% agarose gel. Southern
blot analysis of tail DNA probed with the entire human

10   EC-SOD cDNA was then done. The human EC-SOD cDNA did
not cross-react with the mouse EC-SOD gene. Band
intensity was compared visually to determine which mice
were homozygous, heterozygous, or negative for the human
EC-SOD transgene.

15   ii) Characterization of Transgenic Mice

Northern analysis: Transgenic mice and
nontransgenic littermates were killed with an overdose
of pentobarbital. Tissues were quickly excised and
frozen in liquid nitrogen until ready for further

20   processing. Total RNA was then isolated by the CsCl
procedure was described (Sambrook et al, Molecular
Cloning: A Laboratory Manual. 3. Cold Spring Harbor,
Cold Spring Harbor Laboratory, 1989). Twenty μg of
total RNA from the tissues of transgenic mice and

25   nontransgenic littermates and an RNA ladder were then
denatured with glyoxal, electrophoresed through a 1.2%
agarose gel and blotted to nitrocellulose as described

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(Sambrook et al, Molecular Cloning: A Laboratory
Manual. 3. Cold Spring Harbor, Cold Spring Harbor
Laboratory, 1989). The blots were then probed with the
entire human EC-SOD cDNA.

5   *Separation of SOD isoenzymes by concanavalin A
sepharose chromatography*: Tissues taken from 3 mice
were weighed, then combined and homogenized in
10 volumes of ice-cold 50 mM potassium phosphate, pH
7.4, with 0.3 M KBr, 3 mM diethylenetriaminepentaacetic
10   acid, and 0.5 mM phenylmethylsulfonyl fluoride.
Separation of EC-SOD from CuZn SOD and Mn SOD was
accomplished by passing tissue homogenates over a
concanavalin A sepharose column as described (Marklund
et al, Clin. Chim. Acta 126:4 (1982)).

15   *SOD activity*: EC-SOD activity and total SOD
activity (CuZn SOD and Mn SOD) remaining after EC-SOD
extraction were measured by inhibition of cytochrome C
reduction at pH 10, as previously described (Crapo et
al, Methods Enzymol. 53:382 (1978)). Total protein was
20   determined by the BCA protein assay (Pierce, Rockford,
IL). The SOD activities were then expressed as units/mg
total protein.

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Results:

i)   EC-SOD Transgenic Mice

     Characterization of transgenic mice: Mice carrying
the human EC-SOD transgene were detected by Southern

5   blot analysis. Northern analysis of various tissues
from the F1 of one mouse found to carry the transgene is
shown in Figure 2. High levels of message for human
EC-SOD were detected in the heart, skeletal muscle, and
brain of transgenic mice, with little or no message

10   observed in the lung, liver, and spleen. No message was
detectable in nontransgenic littermates.
     Homozygous mice were generated by breeding two
heterozygous F1 mice. Homozygous mice were detected by
differential band intensities found using Southern blot

15   analysis of equal amounts of PstI digested DNA from the
offspring. EC-SOD activity in the mice was found to
increase in response to the total copies of the EC-SOD
transgene (Table I).

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TABLE I

EC-SOD activity in tissues of nontransgenic,
heterozygous transgenic, and homozygous transgenic mice.
Tissues from 3 mice were combined for each measurement.
5   Activity is expressed as Units/g tissue wet weight.

Tissue	Nontransgenic	Heterozygous	Homozygous
Brain	18	38	50
Heart	35	69	102

Example II

10   Central Nervous System Oxygen Toxicity

Protocols:

     Oxygen exposures: 7-8 week old mice were exposed
to hyperbaric oxygen five at a time in a small-animal
chamber (Bethlehem, Pennsylvania). After flushing the
15   chamber with pure oxygen, compression to 50 meters (6
ATA) was performed within 5 minutes. The oxygen
concentration in the chamber was monitored continuously
with a Servomex oxygen analyzer (model 572, Sybron,
Norwood, Massachusetts) and maintained at ≥99%. The
20   carbon dioxide concentration was analyzed from
intermittent samples of chamber gas with an IR detector
(IR Industries, Santa Barbara, California) and was not
allowed to rise above 0.1%. The chamber temperature

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was maintained at 25-26°C, but the compression of oxygen
in the chamber raised the temperature to 30-32°C
transiently, but an environmental control system
restored the normal chamber temperature within
5   3 minutes. The exposures lasted 25 to 75 minutes and
were followed by decompression for 5 minutes. The mice
were observed continuously for signs of oxygen toxicity
from the beginning of the exposure until 4 hours after
removal from the chamber. The time to the first
10   generalized convulsion (seizure latency) and the time to
death were recorded. These exposure conditions are
designed to cause CNS oxygen toxicity without
appreciable evidence of pulmonary oxygen toxicity.

     Treatment with diethyldithiocarbamate: One hour
15   prior to exposure to 6 ATA oxygen, mice were given
either i.p. injections of either 0.008 cc/g saline or
400 mg/kg diethyldithiocarbamate dissolved in normal
saline (0.008 cc/g). The mice were then exposed to
6 ATA oxygen for 25 mintues as described above.
20   To determine the extent of EC-SOD and CuZn SOD
inhibition by diethyldithiocarbamate, mice were given
diethyldithiocarbamate and sacrificed one hour later.
The brains were removed and assayed for EC-SOD and CuZn
SOD activity as described above.

25   *Treatment with ß-mercaptoethanol*: One hour prior
to exposure to 6 ATA oxygen, mice were given either i.p.
injections of 0.008 cc/g saline or 180 mg/kg ß-
mercaptoethanol (0.008 cc/g). This dose of ß-

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mercaptoethanol was selected because it contains an
equal number of reducing thiols as the dose of
diethyldithiocarbamate. The mice were then exposed to
6 ATA oxygen for 30 minutes as described above.

5   *Treatment with N-ω-nitro-L-arginine, an inhibitor
of nitric oxide synthase*: Ten minutes prior to
beginning compression, 0.008 cc/g saline or 20 mg/kg
(0.008 cc/g) N-ω-nitro-L-arginine dissolved in sterile
water was given i.p to the transgenic and nontransgenic
10   mice. Mice were then exposed at 6 ATA oxygen for 25 or
75 minutes as described above.

        Statistical analysis: A paired Student's t-test
was used to compare enzyme activities in transgenic and
nontransgenic mice. A χ^2 test with Bonferroni
15   correction was used to assess significance in survival
differences to hyperbaric exposures. Analysis of
variance with a Scheffe F-test was used to compare
differences in seizure latency in the different groups
of mice.

20 Results:

        Hyperbaric oxygen exposures: To test the effects
of increased brain EC-SOD levels on CNS oxygen toxicity,
both transgenic and nontransgenic mice (see Example I)
were exposed to 6 ATA oxygen for 25 minutes. Transgenic
25   mice were more susceptible (83% mortality) to CNS oxygen

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toxicity than nontransgenic mice (33% mortality) (Figure
3).

      Transgenic and nontransgenic mice were subsequently
treated with an inhibitor of CuZn SOD,
5   diethyldithiocarbamate, to confirm that the increased
sensitivity of transgenic mice to CNS oxygen toxicity
was the result of increased SOD activity. In both
transgenic and nontransgenic mice, treatment with
400 mg/kg diethyldithiocarbamate resulted in 80%
10   inhibition of EC-SOD and 60% inhibition of CuZn SOD in
the brain. This is consistent with previous findings
(Frank et al, Biochem. Pharmacol. 27:251 (1978);
Heikkila et al, J. Biol. Chem. 251:2182 (1976)).
Treatment with diethyldithiocarbamate conferred
15   increased resistance to CNS oxygen toxicity for both
transgenic and nontransgenic mice. Survival increased
to 100% in transgenic mice and 93% in nontransgenic mice
(Figure 3). The onset of seizures was also delayed
four-fold in mice treated with diethyldithiocarbamate
20   (Figure 4).

      To evaluate whether or not diethyldithiocarbamate
protects against CNS oxygen toxicity by acting as a
reducing agent rather than as an inhibitor of SOD
activity, mice were treated with an equimolar amount of
25   reducing thiols in the form of ß-mercaptoethanol and
exposed to hyperbaric oxygen. Figure 5 shows that ß-
mercaptoethanol did not protect against CNS oxygen
toxicity.

      One possibility that might explain why EC-SOD
30   exacerbates CNS oxygen toxicity would be that nitric

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oxide is a mediator of CNS oxygen toxicity and EC-SOD is
protecting nitric oxide from superoxide mediated
inactivation. To test the hypothesis that nitric oxide
contributes to CNS oxygen toxicity, wild-type

5   (C57BL/6 X C3H)F1 mice were treated with an inhibitor of
nitric oxide synthase, N-ω-nitro-L-arginine. Figure 6
shows the effects of N-ω-nitro-L-arginine on seizure
latency in mice. Pretreatment with N-ω-nitro-L-arginine
resulted in a significant increase in seizure latency

10 (13.50±5.6 min) when compared to saline treated mice
(2.75±1 min). Figure 7 shows that nitric oxide synthase
inhibition also significantly increased survival after
exposure to hyperbaric oxygen. Mice given the inhibitor
of nitric oxide synthase displayed 50% mortality after

15 exposure to 90 minutes of 6 ATA oxygen and 100%
mortality was not obtained until after 2 hours of this
exposure. Saline treated mice, however, had a 50%
mortality after only 25 minutes of exposure, with 100%
mortality after only 30 minutes at 6 ATA of oxygen.

20 Figure 8 shows that the percent survival in hyperbaric
oxygen was dependent on the dose of the inhibitor given.
The protection offered by this competitive inhibitor of
nitric oxide synthase could be reversed when an excess
of L-arginine was given (Figure 6 and Figure 9).

25   The effects of the nitric oxide synthase inhibitor,
N-ω-nitro-L-arginine, upon CNS oxygen toxicity was then
studied in the transgenic mice. This treatment
dramatically reduced CNS oxygen toxicity in both
transgenic and nontransgenic mice. Survival after a

30 25 minute exposure to 6 ATA oxygen increased to 100% in

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both groups (Figure 3). Seizure latency was also
significantly delayed (Figure 4). The exposure time was
then increased to 75 minutes to investigate whether
transgenic mice were still more sensitive than

5   nontransgenic mice to hyperbaric oxygen. The results in
Figure 10 indicate that treatment with N-ω-nitro-L-
arginine abolished the difference in sensitivity that
was observed between untreated transgenic and
nontransgenic mice during the 25 minute exposure shown

10 in Figure 3.

Example III

Cold-Induced Brain Edema

Protocols:

     Injury model: Young (6-7 week) mice (see Example

15 I) were anesthetized with 60 mg/kg pentobarbital
(Nembutal, Abbott Laboratories, Chicago, Illinois). An
incision was then made in the scalp and a steel rod
20 cm long, 3 mm in diameter, equilibrated in liquid
nitrogen with an 8 cm bath of liquid nitrogen 4 cm from

20 the end of the rod, was placed on the skull over the
right cerebral hemisphere for 30 seconds. The skin
incision was then sutured.
     Two hours after the injury the mouse was given an
additional dose of pentobarbital. The chest cavity was

25 opened, the lungs were excised, and the mouse was then
perfused with 20 ml saline through the left ventricle of

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the heart. The brain was then removed and the
cerebellum was excised. The right (R) and left (L)
cerebral hemispheres were separated and immediately
weighed (wet weight, W). Each hemisphere was then dried
5   at 70°C for 2-3 days in a hot air oven until a constant
weight was achieved (dry weight, D). An index of edema
(I) was then calculated as shown in equation 13.

$$I = (W/D \ R - W/D \ L)/(W/D \ L) \ X \ 100 \qquad [13]$$

This calculation allowed the left hemisphere to serve as
10   an internal control for the injured right hemisphere in
each mouse.

     Chemical treatments: Six groups of experiments
were conducted to investigate the importance of
extracellular superoxide, iron, and nitric oxide in
15   cold-induced brain edema. In all groups, drugs were
dissolved in saline and injected at 0.008 cc/g
15 minutes prior to cold injury. In Group 1, edema
formation of EC-SOD transgenic mice was compared with
that of nontransgenic littermates. Group 2
20   edema formation between wild-type (C57BL/6 X C3H)F1 mice
treated with saline and (C57BL/6 X C3H)F1 mice treated
with 0.33 mg/g deferoxamine (0.51 μmoles/g). Group 3
compared (C57BL/6 X C3H)F1 mice treated with saline to
(C57BL/6 X C3H)F1 mice treated with 0.51 μmoles/g Fe^{3+}-
25   saturated deferoxamine. Group 4 consisted of
(C57BL/6 X C3H)F1 mice treated with saline and
(C57BL/6 X C3H)F1 mice treated with 0.02 mg/g N-ω-nitro-
L-arginine methyl ester. Group 5 consisted of
(C57BL/6 X C3H)F1 mice treated with saline and

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(C57BL/6 X C3H)Fl mice treated with 0.02 mg/g N-ω-nitro-
L-arginine methyl ester plus 0.05 mg/g L-arginine.
Group 6 compared edema formation between nontransgenic
mice, EC-SOD transgenic mice treated with saline, and
5   EC-SOD transgenic mice treated with 0.02 mg/g N-ω-nitro-
L-arginine methyl ester.

   Iron saturated deferoxamine was made by dissolving
equimolar amounts of deferoxamine and then ferric
chloride in saline. Saturation of deferoxamine with
10 ferric iron was determined spectrophotometrically be
measuring the absorbance at 425 nm (ε=2500 M^{-1} cm^{-1} for
Fe^{3+}-deferoxamine) (Monzyk and Crumbliss, J. Amer. Chem.
Soc. 104:4921 (1982)).

   Evan's blue treatment: One hour and 50 minutes
15 after cold injury, 5 ml/kg of 1% Evan's Blue in saline
was injected into the femoral artery of transgenic and
nontransgenic mice. The mice were sacrificed 10 minutes
later. The lungs were then excised and the mice were
then perfused with normal saline through the left
20 ventricle until there was no more blue color in the
effluent. The brains were then removed and
photographed.

   Statistical analysis: A paired Student's t-test
was used to compare significance of edema development
25 compared to nontransgenic mice or saline treated mice
for each of the groups examined. Analysis of variance
with a Fisher PLSD test was used to compare significance

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in Group 6. P-value less than 0.05 were considered to
be significant.

Results:

   Cold-induced Brain Edema: When transgenic mice and
5 nontransgenic littermates were subjected to cold-induced
   injury to the right cerebral hemisphere it was found
   that the transgenic mice were significantly protected
   against edema formation compared to nontransgenic
   littermates (Figure 11). Percent edema was 44% less in
10 transgenic mice then in nontransgenic littermates and
   Evan's blue dye extravasation was visibly less in
   transgenic mice compared to nontransgenic littermates
   (Figure 12).
       To test the contribution of iron to edema formation
15 in this model, mice were pretreated with i.p. injections
   of deferoxamine or saline prior to cold-induced injury.
   Table II shows that pretreatment with deferoxamine
   resulted in 43% less edema formation compared to mice
   only given saline. Mice were then pretreated with i.p.
20 injections of iron-saturated deferoxamine or saline
   before cold-induced injury to see if the iron chelating
   properties of this compound were truly necessary for
   protection against edema formation. Table IV shows
   that, even when deferoxamine was saturated with iron, it
25 was still capable of protecting against edema formation
   and resulted in 32-48% less edema than that found in
   saline treated controls. The absolute values for the
   edema index were found to quite variable, however,

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repeated experiments consistently show the same

signficant trends in protection against edema formation

in the various treatments examined.

                            Table II

5   Evaluation of the effect of the ferric iron chelator
       deferoxamine on edema formation after cold-induced brain
       injury. Wild-type (C57BL/6 X C3H)F1 mice were treated
       with deferoxamine to determine what effect the iron
       chelating properties of this compound had on edema
10   formation. Values are presented as mean ± standard
       error.

	Treatment	n	Edema Index
	Saline	7	5.92±0.62
	Deferoxamine	7	3.41±0.49*
Run 1:			
	Saline	6	8.24±0.53
	Fe-Deferoxamine	6	5.63±0.63*
Run 2:			
	Saline	5	7.65±1.30
	Fe-Deferoxamine	5	3.97±0.59*

*$p<0.05$ compared to Edema Index of respective saline
treated controls using a paired Student's t-test.

       These results indicate that deferoxamine is capable

of protecting against edema formation by a mechanism

independent of its ability to scavenge iron. Because

deferoxamine is capable of scavenging both the

peroxynitrite anion (Radi et al, Arch. Biochem. Biophys.

288(2):481 (1991)) as well as the hydroxyl radical (Hoe

                              73

et al, Chemico-Biological Interactions 41:75 (1982)), it
was hypothesized that it is these properties of
deferoxamine that enable it to protect against vasogenic
edema.

5   To test this hypothesis, the synthesis of nitric
oxide was inhibited with N-ω-nitro-L-arginine methyl
ester, a competitive inhibitor of the enzyme nitric
oxide synthase, to determine if this would result in
protection against edema formation after a cold-induced

10   injury. Table III shows that treatment with N-ω-nitro-
L-arginine methyl ester significantly protected mice
against edema formation resulting in 37% less edema
formation than that occurring in saline treated
controls. This protection by N-ω-nitro-L-arginine

15   methyl ester was reversed by simultaneous administration
of an excess of L-arginine to the mice (Table III).

Table III

The effect of inhibition of nitric oxide synthesis on
edema formation after cold-induced brain injury. Wild-
type (C57BL/6 X C3H)F1 mice were treated with the
competitive inhibitor of nitric oxide synthase, N-ω-
nitro-L-arginine methyl ester (LNAME) to determine what
effect nitric oxide had on vasogenic edema. Mice were
also given N-ω-nitro-L-arginine methyl ester plus an
excess of L-arginine (LNAME + L-Arg) to see if the
effects seen with LNAME alone could be reversed. Values
are presented as mean ± standard error.

Treatment	n	Edema Index
Saline	6	5.77 ± 0.29
LNAME	6	3.65 ± 0.51*
Saline	6	6.56 ± 0.21
LNAME + L-Arg	6	6.03 ± 0.71

*$p < 0.05$ compared to Edema Index of respective saline
treated controls using a paired Student's t-test.

     In the final experiments, EC-SOD transgenic mice
were treated with either saline or N-ω-nitro-L-arginine
methyl ester to determine if there was an additive
effect in preventing edema formation in mice which have
both increased levels of EC-SOD as well as the inhibitor
of nitric oxide synthase. Table IV shows that when
EC-SOD transgenic mice were given the inhibitor of
nitric oxide synthase, no added protection against edema
formation was detected relative to transgenic mice
protected only by increased levels of EC-SOD in the
brain.

Table IV

Evaluation of the effect of inhibition of nitric oxide
synthesis on edema formation in transgenic mice.
Comparison of edema formation in nontransgenic mice to
edema formation in transgenic mice with elevated levels
of brain EC-SOD activity, and to edema formation in
transgenic mice treated with an inhibitor of nitric
oxide synthesis (20 mg/kg N-ω-nitro-L-arginine;
Transgenic + LNAME) 15 minutes prior to cold-induced
injury. Values are presented as mean ± standard error
and were compared using analysis of variance with a
Fisher PLSD test. No significant difference was seen
between transgenic and transgenic + LNAME mice.

Treatment	n	Edema Index
Nontransgenic	6	7.91 ± 0.67
Transgenic	6	4.91 ± 0.78*
Transgenic + LNAME	6	4.30 ± 0.96*

*$p < 0.05$ compared to Edema Index of nontransgenic mice.

Example IV

Immunolocalization of EC-SOD

Protocols:

Human lung: Five human lung samples were obtained
to evaluate the distribution of EC-SOD in human lung
tissue. One sample was obtained from a surgical
pathology specimen of a right upper lobe resected from a
43 year old white female with a 50 pack year smoking
history (equivalent to one pack per day for one year)
and a solitary nodule found on chest X-ray. The patient

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was diagnosed with squamous cell carcinoma. Tissue from
a region not involved in the carcinoma from this lobe
was used in the studies presented here. A second lung
was obtained from a right upper lobe surgical pathology
5   specimen resected from a 51 year old white male with a
60 pack year smoking history found to have an isolated
nodule on X-ray. The patient had no other illness and
was diagnosed with squamous cell carcinoma. Lung tissue
not involved in the carcinoma from this specimen was
10   used for the localization of EC-SOD. A third lung was
obtained from a rapid autopsy (tissue obtained 6 hours
after death) of a 66 year old white male with dementia,
but no history of smoking or lung disease. The fourth
lung examined was obtained from excess lung tissue of a
15   lung too large for the recipient of a lung transplant.
The lung was donated from a 45 year old white female
with no history of smoking or lung disease. The fifth
lung examined in these studies was also from excess lung
tissue used for lung transplantation from a 39 year old
20   white male with no history of smoking or lung disease.
Notably, no differences in labeling patterns were seen
between the surgical pathology specimens, the autopsy
tissues from donors for lung transplantation.
        The tissues were fixed in 2% paraformaldehyde/0.2%
25   gluteraldehyde in 0.01 M phosphate buffered saline (PBS;
1.2 g NaH_2PO_4, 8 g NaCl, 0.2 g KCl, in 1 liter pH 7.3)
for 1 hour followed by overnight fixation in 4%
paraformaldehyde at 4°C and then in O.C.T. compound.
The tissues were frozen in liquid nitrogen chilled

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hexane and stored at -70°C until they were sectioned for
light microscopic studies.

   For electron microscopic studies, lung tissues were
processed as in the light microscopic studies up to the
5   equilibration in sucrose. After equilibration in
sucrose, the lung tissues were infiltrated with 10%
gelatin at 37°C for 10 minutes. The tissue slices, in
gelatin, were then solidified on ice, cut into 2 mm/side
cubes, and then cryoprotected in 4% polyvinyl alcohol
10   containing 2 M sucrose overnight. These samples were
then mounted onto stubs, flash frozen in liquid
nitrogen, and then stored in liquid nitrogen until they
were sectioned for electron microscopic studies.

   Characterization of antibody to human recombinant
15   _EC-SOD_: Human recombinant EC-SOD (furnished by S.L.
Marklund, Umeå, Sweden; Tibell et al Proc. Natl. Acad.
Sci. USA 84:6634 (1987)) and the 20,000 x g supernatant
of a human lung homogenate were denatured in the
presence of ß-mercaptoethanol and sodium dodecyl sulfate
20   by boiling for 5 minutes and then electrophoresed
through 12% polyacrylamide gel in the presence of sodium
dodecyl sulfate. The protein was then electrophoreti-
cally transferred to nitrocellulose. The blot was then
incubated with 4.3 μg/ml of an IgG purified fraction of
25   rabbit anti-rh-EC-SOD (furnished by S.M. Marklund, Umeå
University Hospital, Umeå, Sweden) affinity purified
with rh-EC-SOD followed by incubation with ^{125}I-protein
A and autoradiography.

Absorption of anti-EC-SOD IgG: CNBr activated
sepharose was swollen in PBS. Swollen gel was mixed
with PBS so that the settled gel occupied 50% of the
volume. The gel was suspended and 100 μl was mixed with
5   100 μg pure rh-EC-SOD for 2 hours at room temperature
while gently agitating. The gel was then washed 4 times
with PBS + 1% bovine serum albumin (BSA) and made up
100 μl with PBS + 1% BSA. 100 μl of rabbit anti-rh-
EC-SOD at two times the concentration used for
10   immunolabeling was then added and mixed for 2 hours with
gentle agitation at room temperature. Non-immune rabbit
IgG was then added to the supernatant in a concentration
equivalent to the predicted concentration of anti-rh-
EC-SOD IgG removed by the procedure. This supernatant
15   was then used for subsequent immunolabeling.

Light microscopic immunohistochemistry: 4 μm
serial sections of O.C.T. embedded tissue were cut on a
cryostat at -20°C and put on poly-L-lysine-coated slides
(3 sections/slide). Sections were stored at -70°C until
20   labeling was done. Sections were then labeled for
EC-SOD using an indirect immunoperoxidase method (Milde
et al, J. Histochem. Cytochem. 37:1609 (1989); Randell
et al, Am. J. Respir. Cell. Mol. Biol. 4:544 (1991))
with a biotinylated goat anti-rabbit IgG and
25   streptavidin-horseradish peroxidase (Jackson,
ImmunoResearch Laboratoreis (West Grove, Pennsylvania))
(Table V). To reduce background staining, the sections
were incubated in 1% H_2O_2 in methanol to inactivate
endogenous peroxidases, 10 mM borohydride to block

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aldehydes, and nonspecific binding was blocked by
incubation with 5% normal goat serum (NGS), 5% milk, and
1% BSA in PBS. The optimal primary and secondary
antibody dilutions were determined empirically and made

5   in PBS with 1% milk plus 1% BSA (milk was not included
in the streptavidin solution). The slides were
developed using diaminobenzidine (10 mg diamino-
benzidine, 50 ml 0.05 M Tris·Cl, pH 7.6, 100 μl 3% H_2O_2)
and counterstained with 1% methyl green. As a control,

10   serial sections were separately labeled with either
rabbit anti-rh-EC-SOD (EC-SOD), non-immune rabbit IgG,
or rabbit anti-rh-EC-SOD from which EC-SOD binding IgG
had been absorbed out (EC-SOD absorbed; see above).

TABLE V

Staining procedures for light microscopic immunohisto-
chemistry. All incubations were performed in a
humidified chamber at room temperature.

		Incubation Time
Step 1	1% H_2O_2 in methanol	30 minutes
Step 2	10 mM borohydride in PBS (gluteraldehyde fixed tissue only)	30 minutes
Step 3	5% NGS, 5% milk, 1% BSA/PBS 30 minutes	
Step 4	Primary antibody 1% milk, 1% BSA/PBS 1 hour (various dilutions)	
Step 5	Biotin-labeled goat anti-rabbit IgG (1:6000 in 1% milk, 1% BSA/PBS)	1 hour
Step 6	Streptavidin-Horse radish peroxidase (1:2000 in 1% BSA/PBS)	1 hour
Step 7	Diaminobenzidine	15 minutes
Step 8	1% Methyl Green in water	15 minutes

Electron microscopic immunocytochemistry:

Ultrathin cryo sections (70 nm) of human lung tissue

were immunolabeled with rabbit anti rh-EC-SOD and 10-nm

protein A-gold as prevsiously described (Crapo et al,

Proc. Natl. Acad. Sci. USA 89:10405 (1992)) (Table VI).

Briefly, sections were first incubated three times for

five minutes at room temperature in 0.15% glycine in PBS

to block aldehyde groups. Nonspecific binding was

further blocked by incubation in 1% BSA in PBS for

10 minutes. Primary and secondary antibody dilutions

were determined empirically and made in PBS containing

1% BSA. Sections were stained with uranyl oxalate and

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uranyl acetate in methyl cellulose as previously
described (Crapo et al, Proc. Natl. Acad. Sci. USA
89:10405 (1992)). Control groups were as described for
light microscopy above.

5   TABLE VI

Staining procedures for electron microscopic immuno-
histochemistry. All incubations were performed at room
temperature.

	Incubation Time	
Step 1	PBS + 0.15% glycine	
		3 x 5 minutes
Step 2	1% BSA/PBS	
		5 minutes
Step 3	Primary antibody in 1% BSA/PBS 45 minutes	
Step 4	Protein -A gold 30 minutes	
Step 5	Uranyl oxalate	5 minutes
Step 6	Uranyl acetate/methyl cellulose	10 minutes

Results:

        Characteristic of EC-SOD antibody: The antibody to
rh-EC-SOD was characterized by Western blot analysis of
rh-EC-SOD and a human lung homogenate. Figure 13 shows
that the antibody reacted with both the EC-SOD type C
(top band) and the type A (bottom band) subunits
(Sandström et al, Biochem. J. 267:18205 (1992) in a
human lung homogenate. The type A subunit does not
exist in the interstium of tissues in vivo (Sandström et
al, Biochem. J. 290:623 (1993)). The antibody reacted

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with three bands in the lane containing purified type C
rh-EC-SOD. The two lowest molecular weight species in
Figure 13 are due to partial insufficient glycosylation
of the rh-ECSOD in the heavily overproducing CHO-cells.

5   *Light microscopic immunohistochemistry*: Using an
antibody to rh-EC-SOD, this protein was immunolocalized
in human lungs. Light microscopic immunohistochemistry
revealed with EC-SOD is mainly associated with the
connective tissue matrix around vessels and airways in
10   the lung (Figure 14a and b, Figure 15a, b, and c).
EC-SOD was found in close proximity to vascular and
airway smooth muscle (Figure 14a and b, and Figure 15a).
EC-SOD was also seen in connective tissue of alveolar
septal tips (Figure 15c) suggesting an affinity of
15   EC-SOD for connective tissue matrix. No labeling was
seen in association with vascular endothelial cells in
large elastic arteries, medium-sized vessels, or
capillaries, (Figure 14a and b). EC-SOD was notably
absent from the epithelial cell surfaces of airways
20   (Figure 15a and b) and was also not present in cartilage
(Figure 15a).
      The antibody to EC-SOD was an IgG polyclonal rabbit
antibody which was affinity purified using rh-EC-SOD.
To test the specificity of the labeling for EC-SOD, IgG
25   specific for EC-SOD was absorbed out of the antisera
using pure rh-EC-SOD bound to cyanogen bromide
sepharose. Nonimmune rabbit IgG was then added to this
absorbed antibody in a sufficient amount to replace the
absorbed IgG. Labeling lung tissues with this

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preabsorbed antibody preparation resulted in the absence
of labeling in all areas of the lung including the
pulmonary vasculature (Figure 14c). Labeling lung
tissue with nonimmune IgG alone also resulted in the
absence of labeling in all areas of the lung. The
controls indicate that the labeling observed with the
primary antibody is specific for EC-SOD in the lung.

Electron microscopic immunocytochemistry: A
summary of the labeling for EC-SOD in the lung found
using electron microscopic immunocytochemistry is
summarized in Table VII. EC-SOD was mainly associated
with extracellular matrix proteins in all regions of the
lung. In particular, a high degree of labeling was seen
in areas rich in type I collagen (Figure 16) and in
association with other unidentified proteoglycans in
extracellular matrix (Figure 17). Notably, no labeling
for EC-SOD was seen in elastin-rich areas (Figure 16).
A high degree of labeling was observed near the surface
of smooth muscle cells and in the connective tissue
matrix surrounding smooth muscle cells in vessels
(Figure 17) and airways. Labeling was notably absent
from the surface of endothelial cells on small, medium
and large vessels (Figures 18a and b). The lack of
endothelial cell labeling found with the light
microscopic immunohistochemistry support the electron
microscopic findings. Labeling of EC-SOD was also seen
in plasma within the lumen of blood vessels
(Figure 18a). The localization of EC-SOD in plasma is
expected as this protein was first discovered in plasma

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(Marklund, Acta Physiol. Scand., 5492:19 (1980)).
Labeling for EC-SOD was observed in the intercellular
junctions between bronchial epithelial cells
(Figure 19), but was absent from the apical surface of
5   these cells. Finally, EC-SOD labeling was absent from
the surface of type I and type II cells. A moderate,
but consistent amount of intracellular EC-SOD was found
in type II epithelial cells and in bronchial epithelial
cells (Figure 19).

Table VII

Distribution of EC-SOD in human lung. (+) indicates
presence of labeling for EC-SOD and (-) indicates no
labeling for EC-SOD. (±) represents areas where low low
amounts of labeling for EC-SOD were inconsistently
observed.

Location	EC-SOD
Cell Surfaces	
Endothelial	-
Type I cell	-
Type II cell	-
Smooth muscle cell	+
Fibroblast	±
Extracellular Matrix	
Type I collagen	+
Elastin	-
Cartilage	-
Unidentified matrix elements	+
Intracellular	
Endothelial cell	±
Type I cell	-
Type II cell	+
Bronchial epithelial cell	+
Smooth muscle cell	-
Fibroblast	±
Blood	
Plasma	+
Red Blood Cell	-

Controls done by absorbing EC-SOD specific antibody
out of the primary antibody and replacing this absorbed
antibody with nonimmune rabbit IgG resulted in the
absence of labeling in all areas of the lung including
5   areas rich in type I collagen as seen in Figure 16c. In
addition, use of nonimmune rabbit IgG instead of the
primary antisera also resulted in the absence of
labeling in all areas of the lung. The lack of labeling
with these controls indicates that the labeling observed
10 with the primary antisera is specific for EC-SOD in the
lung.

The localization of EC-SOD on the surface of smooth
muscle cells and in the extracellular matrix around
these cells in both blood vessels and airways indicates
15 that EC-SOD may have an important function in this
location. Superoxide is known to rapidly react with
nitric oxide and inactivate its properties as a smooth
muscle relaxant. Therefore, the presence of EC-SOD
along the diffusion path of nitric oxide to smooth
20 muscle cells should increase the half life of this short
lived intercellular messenger in this particular region
and thus increase its potency as a vasodilator. The
high labeling for EC-SOD seen around vascular and airway
smooth muscle cells indicates a function for EC-SOD as a
25 mediator of nitric oxide activity in the maintenance of
low pulmonary vascular pressures and airway resistence.

In addition to the labeling of EC-SOD in
association with smooth muscle cells, EC-SOD also
appears to strongly colocalize with type I collagen.

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Collagen has previously been demonstrated to be
susceptible to attack by reactive oxygen species such as
the superoxide anion. In addition, the superoxide anion
may be capable of activating latent collagenases from
5   polymorphonuclear leukocytes (PMN) which can lead to
further collagen degradation. Because collagen
fragments have been shown to chemoattract and activate
PMN's, any increased produced of superoxide that results
in collagen degradation may accelerate inflammatory
10   reactions and tissue destruction through PMN recruitment
and activation. Consequently, the association of EC-SOD
with collagen may be important in preventing superoxide
mediated degradation of collagen and therefore,
represent a means of controlling inflammatory responses.

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Example V

Human EC-SOD Gene

Protocols:

   Materials and radiochemicals:

5   [α-^{35}S]dATP (~1400 Ci/mmol), [γ-^{32}P]ATP (3000

Ci/mmol), and [α-^{35}P]CTP (800 Ci/mmol), were purchased

from New England Nuclear. Human genomic DNA, T_7, T_3,

and SP6 RNA polymerase, RNasin, and the pGEM3Zf(+)

plasmid were obtained from Promega Biotec. Sequenase

10   sequencing kit (V 2.0) was from United States

Biochemicals Corporation. Human poly A+ RNA was

acquired from Clontech. SeaPlaque GTG agarose was from

FMC BioProducts. Restriction enzymes were from New

England Biolabs. All other reagents used were molecular

15   biology grade. Oligonucleotides were synthesized using

an Applied Biosystems 380B or 392 by the Duke

University, Department of Botany DNA synthesis facility.

Charged nylon membranes (GeneScreen Plus) were from

DuPont.

20   Human Northern blot or analysis:

   Two μg poly A+ RNA were purified from eight

different human tissues. These mRNAs were

electrophoresed on a denaturing formaldehyde 1.2%

agarose gel and transferred to a charge-modified nylon

25   membrane followed by UV irradiation fixation. The

membrane was prehybridized in 50% formamide, 0.25 M

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NaPO$_4$ (pH 7.2), 0.25 M NaCl, 1 mM EDTA, 7% SDS, and 5%
polyethylene glycol (molecular weight 8000). The blot
was hybridized in the same buffer overnight at 60°C with
1 X 10^6 cpm/ml of [^{32}P]-labeled human EC-SOD RNA
5   generated by transcription of the full-length cDNA using
T$_3$ RNA polymerase in the presence of [α-^{32}P]CTP. The
blot was washed in 0.25 M NaPO$_4$ (pH 7.2), 1% SDS, and
1mM EDTA at 75°C followed by a second wash using 0.04 M
NaPO$_4$ (pH 7.2), 1% SDS, and 1 mM EDTA at 75°C for
10   30 minutes. This was followed by exposure to XAR-5 film
using a Lightening Plus intensifier screen at -70°C.
The autoradiogram was scanned using an LKB Ultrascan XL
laser densitometer, and the peaks were quantitated by
integration using the internal digital integrator of the
15   densitometer or by cutting out the peak from a printer
tracing and weighing.

      5' Rapid amplifciation of cDNA ends:

      0.5 μg of poly A+ mRNA from human heart was
reversed transcribed using 2 pmoles of EC7, a 5' EC-SOD
20   gene specific anti-sense oligonucleotide (5'-
ATGACCTCCTGCCAGATCTCC-3'), following a protocol by GIBCO
BRL (5' RACE System). The RNA template was degraded by
the addition of RNase H at 55°C for 10 minutes. The
resulting cDNA was isolated using a GlassMAX DNA (GIBCO
25   BRL) isolation spin cartridge. The purified cDNA was
dC-tailed using terminal deoxynucleotidyl transferase
(TdT, 0.5 units/ul). 200 μM dCTP, 10 mM Tris-HCl (pH
8.4), 25 mM KCl, 1.25 mM MgCl$_2$ and 50 μg/ml bovine serum

albumin for 10 minutes at 37°C. The TdT was heat

inactivated for 10 minutes at 70°C.

   Products of this reaction were next PCR amplified

using the "anchor" primer (GIBCO BRL), which hybridizes

5   to the homopolymeric tail, and EC4 (a nested internal 5'

EC-SOD gene specific anti-sense oligonucleotide,

5'-AGGCAGGAACACAGTAGC-3'). Alternatively, the dC-tailed

products were PCR amplified using EC7 and HEC1 (a sense-

strand EC-SOD gene specific primer,

10   5'-TGGGTGCAGCTCTCTTTTCAGG-3'). The final composition of

the reaction included 20 mM Tris-HCl (pH 8.4), 50 mM

KCl, 2.5 mM $MgCl_2$, 100 $\mu g/ml$ bovine serum albumin, 400

nM Anchor primer, 200 nM gene-specific primer, and 200

μM each of dATP, dCTP, dGTP, dTTP. After incubating the

15   PCR reaction for 5 minutes at 94°C, amplitaq (Perkin

Elmer Cetus) was added at a final concentration of 0.04

units/μl. PCR cycling was performed on a Perkin Elmer

9600 for 35 cycles with melting at 94°C for 45 seconds

and annealing at 53°C for 15 seconds and extension at

20   72°C for 90 seconds. The full-length EC-SOD cDNA (6 ng)

was used as a positive control in the PCR reaction. The

PCR products were electrophoresed in a 2% SeaPlaque GTG

agarose gel, transferred to charged nylon membranes by

the method of Southern (Southern, J. Mol. Biol. 98:503

25   (1975)) using the alkaline transfer protocol (Reed et

al, Nuc. Acids Res. 13:7207 (1985)). The DNA was fixed

to the membrane by baking at 80°C in a vacuum oven for

2 hours. The subsequent blot was hybridized to [^{32}P]

end-labeled HEC2 (an internal, nested EC-SOD specific

30   primer, 5'-TCCAGCTCCTCCAAGAGAGC-3') overnight at 37°C.

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The blot was washed at increasing stringencies until
background hybridization was removed. This was followed
by exposure to XAR-5 film using a Lightening Plus
intensifier screen at -70°C.

5   _Human genomic Southern blot analysis:_

     Ten μg human genomic DNA were digested BamH I,
EcoR I, Kpn I, and Pst I restriction endonuclease
enzymes until completion. The DNA was then
electrophoresed on 1% agarose gel and transferred to a
10   charged nylon membrane by the Southern technique
(Southern, J. Mol. Biol. 98:503 (1975)), after alkaline
denaturation (Reed et al, Nuc. Acids Res. 13:7207
(1985)). The DNA was fixed to the membrane by heating
to 80°C in a vacuum oven for 2 hours. $[^{32}P]$CTP-labeled
15   human EC-SOD antisense strand cRNA was synthesized using
the EC-SOD cDNA which had been linearized with Stu I.
The blot was hybridized (500 X 10^3 cpm/ml) in 50%
formamide, 0.25 M $NaPO_4$ (pH 7.2), 0.25 M NaCl, 1 mM
EDTA, 7% SDS, and 5% polyethylene glycol (molecular
20   weight 8000), at 50°C. Following overnight
hybridization, they were washed in 0.25 M $NaPO_4$ (pH
7.2), 2% SDS, and 1 mM EDTA followed by 0.04 M $NaPO_4$ (pH
7.2), 1% SDS, and 1 mM EDTA at increasing stringencies
until background hybridization was minimized. The blot
25   was exposed to XAR-5 film using a Lightening Plus
intensifier screen at -70°C.

Isolation of the human gene for EC-SOD:

A human adult female leukocyte genomic library
constructed in the EMBL-3 vector was obtained from
Clontech. Approximately 1 X 10^6 pfu were screened at a
5   density of ~50,000 pfu/plate using [^{32}P]CTP-labeled
human EC-SOD cRNA (1 X 10^6 dpm/ml). The primary through
tertiary screens identified approximately 7 unique
putative positive plaques. Individual plaques were
isolated and lambda DNA purified using LambdaSorb phage
10   adsorbent (Promega Biotec). The size of the insert DNA
from each clone was assessed by Sal I restriction
endonuclease digestion followed by electrophoresis in
0.7% agarose. Selected clones underwent extensive
restriction endonuclease mapping. Based on the
15   restriction mapping results and asymmetric hybridization
using 5' and 3' annealing EC-SOD oligonucleotides, Clone
#7 was selected for all subsequent DNA sequence
analysis. Clone #7 contains an approximate 18-20 kb
fragment.

20   *DNA sequencing of the human EC-SOD gene*:

The overall strategy used for sequencing clone #7
is illustrated in Figure 20. Various size restriction
endonuclease DNA fragments from clone #7 were subcloned
into the pGEM3Zf(+) vector DNA. The dideoxy sequencing
25   method using double-stranded DNA (Ausubel et al, Current
Protocols in Molecular Biology, Green Publishing Assoc.
and Wiley Interscience, New York (1992)) as template and
Sequenase enzyme (United States Biochemicals) were
employed (Sanger et al, Proc. Natl. Acad. Sci. USA

74:5463 (1977)). Both the Universal and -40 M13
sequencing primers were used to initiate DNA sequencing
for each subcloned fragment. Oligonucleotides derived
from this initial sequencing data were synthesized
5   approximately every 250 base pairs until the complete
nucleotide sequence was obtained. Sequencing data were
obtained from both strands as shown in Figure 20B except
at the 3' portion of the gene where DNA sequence was
obtained on one strand only.

10   _Computer-assisted sequence analysis and_
transcriptional database search:
        The IntelliGenetics Geneworks program (Version 2.2)
was used for organizing the DNA sequence data. Homology
searching was performed at the NCBI using the BLAST
15   (Altschul et al, J. Mol. Biol. 215:403 (1990)) network
service and the non-redundant nucleotide sequence
database (GenBank(77.0) + EMBL(35.0) + EMBLUpdate +
GBUdate). Transcriptional factor database searching was
performed using both the SIGNAL SCAN 3.0 algorithm
20   (Prestridge et al, CABIOS 9:113 (1993)) as well as the
FINDPATTERNS program of the GCG Package (V 7.2) using
Release 6.3 of the Transcription Factor Database (Gosh,
Nuc. Acids Res. 18:1749 (1990)). For a prediction of
the site of signal peptide cleavage, the programs
25   SIGSEQ1 (Folz et al, J. Biol. Chem. 261:14752 (1986))
and SIGSEQ2 were employed (Folz et al, Biochem. Biophys.
Res. Commun. 146:870 (1987)).

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Results:

Tissue specific expression of human EC-SOD:

To investigate the expression of human EC-SOD, poly
A(+) mRNA from eight different human tissues was
5   fractionated on a denaturing agarose gel and transferred
to a charged nylon membrane. Because a previous paper
reported long exposures times in order to identify EC-
SOD specific bands during genomic Southern analysis
(Hendrickson et al, Genomics 8:736 (1990)), a
10 radiolabeled antisense cRNA probe derived from full-
length human EC-SOD cDNA was used (Oury et al, Proc.
Nat. Acad. Sci. USA 89:9715 (1992)). A discrete band of
approximately 1.4 kb can be seen in all eight human
tissues analyzed (Figure 21A). In addition, skeletal
15 muscle contains an approximate 4.2 kb message, not
detected in the other tissues. By densitometric
scanning of the 4.2 and 1.4 kb bands, it can be
calculated that larger message to make up about 32% of
the total skeletal muscle EC-SOD message. In brain, a
20 very faint band of 2.2 kb can be seen. This band was
too weak for quantitation by laser densitometer.
Quantitation of these bands was performed by laser
densitometry and integration of peaks of autoradiograms
obtained in the linear range of exposure (Figure 21B).
25 After normalizing to the brain, the heart showed the
most expression with 10.1 times brain. This was
followed by the placenta, pancreas, and lung which gave
13.6, 10.2, and 7.5, respectively. Expression in
skeletal muscle was 4.7 for the 1.4 kb band or 6.9 for

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both 1.4 kb and 4.2 kb message, while the kidney and liver gave 6.3 and 4.1 time expression over brain. These patterns of expression have been reproduced based on probing an additional independent multiple tissue

5   Northern blot. The bands are specific based on the relatively high stringencies of washing and from data using a sense strand EC-SOD cRNA as a probe which showed no hybridization under the conditions given.

Mapping the site transcription initiation:

10   Initially, mapping of the site of transcription initiation was attempted using the primer extension method. Using several different end-labeled 5' oligonucleotides and both human lung and human heart poly A+ mRNA as well as total RNA isolated from human

15   foreskin fibroblasts, a positive signal was not obtained even after long exposure times. This did not seem to be due to technique since it was not possible to get positive signals using RNA generated by _in vitro_ transcription of the EC-SOD cDNA. Whether lack of

20   success using this technique was due to very low abundance of mRNA encoding EC-SOD or some other problem(s) is unclear. Working under the assumption of low abundance mRNA, the technique of rapid amplification of cDNA ends in order to PCR amplify this signal was

25   attempted. The EC-SOD gene specific primer EC7 was used for hybridization and reverse transcription of human heart poly A+ mRNA as shown in Figure 22. Half of this reaction was 3' dC tailed using terminal deoxynucleotidyl transferase and the remaining half was

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not. These templates were then subjected to PCR
amplification using the gene specific primers HEC1 + EC7
as well as the anchor primer + EC4. The products of
these reactions were fractioned by agarose
5   electrophoresis, transfered to nylon membranes, and
probed with the interal nested gene specific primer
HEC2. An autoradiogram of this experiment is shown in
Figure 22A. Using EC-SOD cDNA as a control template and
HEC1 + EC7, a band of 217 bp is expected (lane 3 of
10   Figure 22A). Since the primers HEC1 and EC7 are
expected to amplify independant of dC tailing, bands of
equal intensity in lanes 4 and 5, which are also of the
same size as the EC-SOD control, are seen. Using the
anchor primer (which hybridizes to the dC tail) and EC4,
15   only one band of ~190 bp was seen (lane 1). Since the
template was not poly C tailed, lane 2 shows no signal
as expected. By subtracting 48 bp (the size of the
anchor primer), the size of the reverse transcribed DNA
would correspond to ~136 bp 5' of the EC4 primer. This
20   analysis would predict that there is approximately 6
base pairs of additional 5' sequence on the cDNA clone
and that transcription initiation starts about 6 bp
upstream of the first intron (indicated by a dashed
box). Although eukaryotic transcription initiation
25   usually begins at an adenosine residue, it is expected
that it will begin at a G (Breathnach et al, Ann. Rev.
Biochem. 50:349 (1981)).

Genomic Southern blot analysis:

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To begin to charcterize the human EC-SOD gene, 10
μg of the total human genomic DNA was restriction
digested and the reaction products electrophoresed on an
agarose gel followed by transfer to a nylon membrane.

5   The blot was probed with a [^{32}P]-labeled partial EC-SOD
cRNA. An autoradiogram of this blot is shown in Figure
23. As can be seen for each lane, there are unique
bands associated with each restriction digest. No
shadow bands which might suggest pseudogenes were seen.

10   When a full-length cRNA probe was used for Kpn I
digested DNA, an additional band of ~4000 bp was seen
which corresponds to the 3' end of the gene. In
addition, the Kpn I lane shows a 0.5 kb band which was
better seen on other blots. This banding pattern was

15   similar to a restriction map of the human EC-SOD clone
#7 (see Figure 20A).

   Isolation and characterization of the human EC-SOD
by DNA sequencing:

   Multiple independent positive clones were

20   identified from a human adult leukocyte genomic library
constructed in EMBL-3. These clones underwent extensive
restriction endonuclease mapping and were probed with
EC-SOD specific 5' and 3' oligonucleotides in order to
determine the relative orientation of the inserts.

25   Based on these results, clone #7 was picked for further
analysis. Clone #7 is about 18 to 20 kb and contains at
least 5000 bp of 5' flanking DNA and at least 4000 bp of
3' flanking DNA. Restriction mapping of clone #7 is
shown in Figure 20A. This map is similar to the results

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obtained with genomic Southern blot analysis data
indicating that clone #7 contains the EC-SOD gene. The
strategy for sublconing and sequencing clone #7 is shown
in Figure 20B. Various size contiguous and overlapping
5   restriction fragments were subcloned into the plasmid
vector pGEM32f(+) (Figure 20B). The DNA inserts were
sequenced on both strands using a combination of primer
walking and vector specific, universal sequencing
primers. The 3' half of 7K36 insert was sequenced on
10   one strand only. Published sequence data for the human
EC-SOD cDNA (Hjalmarsson et al, Proc. Natl. Acad. Sci.
USA 84:6340 (1987)) as well as DNA sequence information
obtained from an independant cDNA clone which contained
additional 5' untranslated data (Hendrickson et al,
15   Genomics 8:736 (1990)) were used to determine the
genomic intron/exon structure. Based on a comparison of
these data with the genomic sequence information, the
human EC-SOD gene was determined to contain three exons
and two introns (Figure 20C). Exon 1 contains at least
20   5 base pairs and is probably larger (by about 6 base
paris), since the exact start of transcription
initiation was not determined (note below). Exon 2
contains 84 bp and is separated from exon 1 by a 572 bp
intervening sequence marked as intron 1. Exon 3 is
25   separated from exon 2 by intron 2, a 3849 bp segment.
Exon 3 contains a total of 1336 bp and at 17 bp into
this exon starts the beginning of the complete coding
sequence for preEC-SOD (Figure 20D). This includes an
18 amino acid signal peptide that precedes the 222 amino
30   acid mature protein sequence. There are no introns

separating the various structural domains of EC-SOD.
These domains are shown schematically in Figure 20D and
include amino acids 1-95 which contain a glycosylated
Asn-89 and show no sequence homology with other
5   proteins. Resides 96-193 show strong homology with
CuZn-SOD protein sequences with preservation of critical
amino acids important in enzyme catalysis and structure.
Amino acids 194-222 contain multiple charged resides
which have been shown to be important in binding to
10   sulfated proteoglycans. 558 bp of the 5'-flanking
region containing putative regulatory elements and 3675
bp of the 3'-flanking region were also sequenced. The
exonic DNA sequence data are in agreement with the
published cDNA sequence (Hjalmarsson et al, Proc. Natl.
15   Acad. Sci. USA 84:6340 (1987)). The intron-exon
boundries are shown in Table VIII and conform to the
eukaryotic consensus splice sequence (Senapathy et al,
Methods Enzymol. 183:252 (1990)). Both introns split
sequences in the 5'-nontranslated region of the EC-SOD
20   gene.

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Table VIII

Sequences at intron/exon splice junctions

The size of the introns and exons are shown in base
pairs (bp). The uppercase letters indicate exon
5   sequence while the lowercase letters indicate intron
sequence. The splice junctions shown conform to
previously published concensus sequences for splice
junctions (Senapathy et al, Methods Enzymol. 183:252
(1990)).

Donor	Intron size (bp)	Acceptor	Exon
TGCGGG gt ggac	572	gccc ag GCTCCA	84
GGAAAG gt gggt	3849	ccgc ag GTGCCC	1336

Figure 24 shows the entire sequence for the human

15   EC-SOD gene. Exonic sequences are shown in boxed

uppercase letters while intronic, 5'- and 3'-flanking

sequence are shown in lowercase. Exon 3 contains the

entire uninterrupted coding region for EC-SOD and the

protein sequence is shown using the single letter amino

20   acid code. The 18 amino acid signal peptide and 222

amino acid mature protein sequence are highlighted. The

identification of the signal peptide cleavage site is

consistent with computer algorithms which predict the

site of eukaryotic signal peptide cleavage (Folz et al,

25   Biochem. Biophys. Res. Comm. 146:870 (1987)); Von

Heijne, Eur. J. Biochem. 133:17 (1983)).

Transcriptional factor database searching was used

to putatively identify transcriptional regulatory

elements. Although almost all eukaryotic promoters

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utilize a TATA box element to fix the position of
transcription initiation, an obvious TATA box cannot be
discerned for the EC-SOD gene. Two CAAT box elements
were identified. One is in the reverse orientation and
5   located about 20 bp upstream of the first exon, while
the second can be found about 335 bp upstream. The
putative signal for polyadenylation is shown and the
site of poly A adenylation is indicated.
Transcriptional factor database searching of the 5'-
10   nontranslated region and first intron identified several
potential regulatory elements. A cAMP responsive
element (CREB) (TGACGT) which is similar to the
adenovirus transcription virus (ATF) element can be
found starting at 121 bp (Fink et al, Proc. Natl. Acad.
15   Sci. USA 85:6662 (1988); Sassone-Corsi Proc. Natl. Acad.
Sci. USA 85:7192 (1988)). A half site for the
glucocorticoid response element (GRE) (TGTCCT) is
located at 370 bp (Karin et al, Nature 308:513 (1984)).
A skeletal muscle specific trans-activating factor
20   response element (M-CAT) (CATTCCT) is found in the
reverse orientation beginning at position 238 (Mar et
al, Mol. Cell. Biol. 10:4271 (1990)). A xenobiotic
responsive element (XRE) (CACGCW) is found within the
first intron at position 1085 bp (Rushmore et al, J.
25   Biol. Chem. 265:14648 (1990)). A metal regulatory
element (MRE) (TGCRCYC) is found at position 89 (Culotta
et al, Mol. Cell. Biol. 9:1376 (1989)). Two putative
antioxidant response elements (ARE) (RGTGACNNNGC) are
found at position 650 and 5022 (Rushmore et al, J. Biol.
30   Chem. 266:11632 (1991)). A sis responsive element (SIF)

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(CCCGTC) important in the induction of the c-fos proto-
oncogene is found in the reverse orientation at position
251 (Wagner et al, EMBO J. 9:4477 (1990)). There is an
AP1 binding site or TPA responsive element (TRE)
5   (TGACTCA) found at position 162 (Risse et al, EMBO J.
8:3825 (1989)). The SV40 enhancer region AP4
(CAGCTGTGG) can be found at position 171 (Jones et al,
Genes Dev. 2:267 (1988)).

                         Example VI

10   Screening Patients for Gene Defects in EC-SOD

     Preparation of leukocyte derived genomic DNA from
     patients: Normal healthy control patients and patients
     with asthma, primary pulmonary hypertension, and
     secondary pulmonary hypertension will be identified.
15   Genomic DNA will be purified utilizing a Qiagen Blood
     PCR Kit. One ml of blood containing ~10^7 leukocytes/ml
     will be collected in sodium citrate from each patient or
     control subject. The blood is placed into a QIAGEN-spin
     column and the leukocytes are entrapped in the resin by
20   brief centrifugation, while erythrocytes and hemoglobin
     are washed through. The leukocytes are lysed by the
     addition of 0.7 ml of lysis buffer and incubated at room
     temperature for 30 minutes. DNA that is released, binds
     to the resin in the tube. Remaining cellular debris is
25   washed away by multiple wash/spin cycles. The DNA is
     eluted by the addition of 1.2 M KCl, 50 mM MOPS, 15%
     ethanol, pH 8.3. This typically yields ~10 μg of

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genomic DNA (Reihsaus et al, Am. J. Respir. Cell. Mol.
Biol. 8:334 (1993)).

Primer design and PCR amplification of EC-SOD exonic
sequences: Sense and antisense oligonucleotide primers

5   (or use primers already obtained from sequencing the
genomic DNA) will be designed containing a 3'GC clamp
(Sheffeld et al, Proc. Natl., Acad. Sci. USA 86:232
(1989)). These primers will encode the intronless
coding region of the EC-SOD gene. A 172 bp region in

10   the 3' untranslated region has been amplified using DNA
sequencing primers and human genomic DNA. PCR
conditions are as described (Reihause et al, Am. J.
Respir. Cell. Mol. Biol. 8:334 (1993); Innis et al (eds)
Academic Press San Diego pp. 1-12 (1990)) using Taq

15   polymerase, with temperature cycling as follows:
initial denaturation at 95°C for 5 min followed by
35 cycles of 94°C denaturing for 20 sec, 57°C annealing
for 15 sec, and 72°C elongation for 45 sec. Because of
the GC composition and actual primer sequence, it will

20   be necessary to experimentally optimize conditions for
PCR amplification using each set of primers. Three sets
of primers will be used to encompass the entire coding
region.

Identification of mutations with single-strand

25   conformational polymorphism (SSCP) analysis: SSCP
analysis has been used to detect single base pair
mismatch (Orita et al, Genomics 5:874 (1989)).
Temperature-gradient gel electrophoresis (TGGE) will be

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used to detect differences in mobility (Wartell et al,
Nuc. Acids Res. 18:2699 (1990)). Samples for TGGE will
be prepared by heat denaturing the PCR product at 98°C
for 5 min, then renaturing at 50°C for 15 min with the
corresponding wild-type DNA derived from PCR of the
cloned gene. Electrophoresis will be carried out on a
5% acrylamide, 8 M urea gel over a temperature gradient.
The temperature gradient will be optimized for each of
the EC-SOD DNA segments. Typical gradients for the
detection of ß$_2$-adrenergic receptor mutations were
between 35°C to 60°C, and required 4 to 6 hours of run
time (Rosen, Nature 262:59 (1993)).

    All PCR samples found to be positive for mutations
by TGGE will be sequenced directly using the dideoxy
technique (Sanger et al, Proc. Natl. Acad. Sci. USA
74:5463 (1977)).

                        Example VII

               Inhibition of Xanthine Oxidase

    In a first study, assays were performed in a 1 ml
quartz cuvette containing 50 mM carbonate buffer, pH 10,
0.1 mM EDTA, 1 nM xanthine oxidase (Boehringer Mannheim)
at 25°C. Xanthine oxidase activity was measured
spectrophotometrically by following the loss of xanthine
over time at 295 nm. Four concentrations of xanthine
(25, 50, 250, 500 µM) and 2 concentrations of MnTBAP (5,
10 µM) were used. Two inhibition constants were then
derived from the curve's intercepts (Kii=5.5 µM) and

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slopes (Kis=15 μM). The results presented in Figure 25
show that MnTBAP inhibits xanthine oxidase in a non-
competitive manner.

In a second study, calf pulmonary artery
5   endothelial cell cultures (CPA-47 (Tissue and Cell
10:535 (1978)) were grown to confluence in Ham's F-12K
medium with 10% fetal bovine serum at pH 7.4 and 37°C.
Cells were then trypsinized and seeded at equal
densities in 24 well plates and grown to 90% confluence.
10   Cells were washed and pre-incubated for 1 hour with
50 μM of MnTBAP in minimum essential medium (MEM) or MEM
only. Varing amounts of xanthine oxidase (XO) plus
200 μM xanthine (X) were added and allowed to incubate
for 24 hours. Cell injury was quantitiated by measuring
15   the release of cellular lactate dehydrogenase (LDH) into
the medium. The efficacy of MnTBAP is shown in Figure
26 by the decrease in XO/X-induced LDH release.

Example VIII

SOD Mimetic Affords Cellular Protection
20   From Paraquat-Induced Injury

Rat pulmonary epithelial cell cultures (L2 (Kaighn
and Douglas J. Cell Biol. 59:160a (1973)) were grown to
confluence in Ham's F-12K medium with 10% fetal bovine
serum at pH 7.4 and 37°C. Cells were then trypsinized
25   and seeded at equal densities in 24 well plates and
grown to 90% confluence. Cells were washed and pre-
incubated for 1 hour with 100 μM of MnTBAP or MnTMPyP in

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MEM or MEM only. Paraquat (2.5 mM) was added and
allowed to incubate for 48 hours. Cell injury was
quantitiated by measuring the release of cellular
lactate dehydrogenase (LDH) into the medium. Figure 27

5   shows that MnTPyP (hatched bars) and MnTBAP (grey bars)
decrease paraquat-induced LDH release.

In a further study, calf pulmonary artery
endothelial cell cultures (CPA-47 (Tissue and Cell
10:535 (1987)) were grown to confluence in Ham's F-12K

10 medium with 10% fetal bovine serum at pH 7.4 and 37°C.
Cells were then trypsinized and seeded at equal
densities in 24 well plates and grown to 90% confluence.
Cells were washed and pre-incubated for 1 hour with
varing concentrations of MnTBAP in MEM or MEM only.

15 Paraquat (2 mM) was added and allowed to incubate for
24 hours. Cell injury was quantitiated by measuring the
release of cellular lactate dehydrogenase (LDH) into the
medium. MnTBAP decreases paraquat-induced LDH release
in a dose-dependent manner (see Figure 28).

20 In contrast to MnTBAP, ZnTBAP does not protect
against paraquat-induced injury. Calf pulmonary artery
endothelial cell cultures (CPA-47) were grown to
confluence in Ham's F-12K medium with 10% fetal bovine
serum at pH 7.4 and 37°C. Cells were then trypsinized

25 and seeded at equal densities in 24 well plates and
grown to 90% confluence. Cells were washed and pre-
incubated for 1 hour with varing concentrations of
ZnTBAP in MEM or MEM only. Paraquat (2 mM) was added
and allowed to incubate for 24 hours. Cell injury was

30 quantitiated by measuring the release of cellular

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lactate dehydrogenase (LDH) into the medium. The
results presented in Figure 29 demonstrate that ZnTBAP
does not possess SOD-like activity. ZnTBAP can be used
as a negative control to show that the redox metal is
5   important in the protection against paraquat toxicity.

Example IX

Protection by MnTBAP Against
Paraquat-Induced Lung Injury

Mice were treated with either paraquat (PQ,
10   45 mg/kg, ip) or saline (10 ml/kg, ip) and exposed to
MnTBAP (2.5 mg/ml, nebulized into a 2 L chamber at
2 L/min for 30 minutes twice daily for 2 days) or room
air. Mice were killed 48 hours after start of treatment
and lung injury was assessed by analysis of
15   bronchoalveolar lavage fluid (BALF). BALF damage
markers used were lactate dehydrogenase (LDH, as
units/L), protein concentration (as mg/dl), and percent
of polymorphonuclear leukocytes (PMN). MnTBAP treatment
provided partial protection against paraquat-induced
20   lung injury (see Figure 30).

Example X

Catalase activity was measured by means of a Clark
oxygen electrode using a modified assay previously
described by Del Rio et al, Anal. Biochem. 80:409
25   (1977). Briefly, reactions were performed in a nitrogen

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degassed phosphate buffer (50 mM, pH 7.8) containing
0.1 mM EDTA at 25°C. Three concentrations of hydrogen
peroxide (1-4 mM) and four metalloporphyrin
concentrations (0.5-50 μM) were used to determine second
5   order rate constants. The rate of oxygen evolution was
followed for 2 minutes. The results are shown in
Figure 31.

Calf pulmonary endothelial cell (CPA-47) line was
grown to near confluence in 12-well plates with F-12K
10 medium containing 10% fetal calf serum. CPA-47 cells
were loaded with Cr^{51} as previously described by Simon et
al J. Clin. Invest. 78:1375 (1986) in high glucose
minimum essential medium (DMEM). Cells were pretreated
with MnTBAP (100 μM) for 1 hour and then exposed to
15 various concentrations of hydrogen peroxide generator,
glucose oxidase, for 4 hours. Cell injury was then
quantitated as the specific Cr^{51} release from CPA-47
cells which has been adjusted for spontaneous Cr_{51}
release. The results are shown in Figure 32.

20 Calf pulmonary endothelial cell (CPA-47) line was
grown to near confluence in 12-well plates with F-12K
medium containing 10% fetal calf serum. CPA-47 cells
were loaded with Cr^{51} as previously described by Simon et
al (J. Clin. Invest. 78:1375 (1986)) in high glucose
25 minimum essential medium (DMEM). Cells were pretreated
with various concentrations of either: (A) MnTBAP; (B)
MnTMPyP; (C) ZnTBAP; or (D) CuZnSOD for 1 hour and then
exposed to the hydrogen peroxide generator, glucose
oxidase (100 Mu/ml), for 4 hours. Cell injury was then
30 quantitated as the specific Cr^{51} release from CPA-47

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cells which has been adjusted for spontaneous Cr^{51} release. The results are shown in Figure 33A-33D.

Example XI

Mimetics as Protectants Against Excitotoxic Cell Death

5   Experimental procedures

Tissue culture: Mixed neuronal and glial cultures were prepared from embryonic day-18 rat cerebral cortices (Sprague-Dawley, Zivic Miller). Briefly, the cerebral cortices were dissected and enzymatically

10   dissociated by incubation in Ca^{++}-, Mg^{++}-free Hank's balanced salt solution (HBSS) supplemented with 10 mM HEPES and 0.25 % trypsin for 20 min. at 37°C. The tissue was rinsed and dispersed into single cell suspension by gentle passage through a fire polished Pasteur pipette.

15   The cell suspension was centrifuged and resuspended in Minimum Essential Media (MEM), containing Earle's salts supplemented with 3 g/L glucose, 5% horse serum and 5% fetal bovine serum (growth media). The cells were plated in poly-D-lysine-coated multi-well plates: 12-well

20   plates for aconitase measurement, and 24 well plates for toxicity experiments. Cells were maintained at 37°C in a humidified incubator with 5% CO_2/95% air in growth media. Media was not replaced so as to reduce glial overgrowth and neuronal loss. Mature cells (14-17 days

25   *in vitro*) were used for all experiments.

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   Cell treatment: The growth media was replaced with
MEM supplemented with 25 mM glucose (MEM-g). For both
neurotoxicity studies and aconitase measurement, cells
were incubated in the designated treatment for the
5   indicated length of time at 37°C. Unless otherwise
specified, antagonists or SOD-mimetics were added 15
min. prior to agonists. For measurement of
neurotoxicity, cells were incubated with treatments for
18 hr at 37°C in MEM-g. To assess the ability of
10 antagonists to inhibit acute NMDA toxicity or rescue
cells from an ongoing NMDA insult, an alternate paradigm
of NMDA treatment was used in addition to the one
described above. In this paradigm, growth media was
replaced with HBSS⁻ (Ca⁻⁻, Mg⁻⁻-free Hank's Balanced Salt
15 Solution supplemented with 2 mM $CaCl_2$, 1 mM $NaHCO_3$, 10 mM
HEPES and 5 µM glycine), cells were treated with vehicle
or 100 µM NMDA for 15 min. after which HBSS⁻ was replaced
with MEM-g and cells were returned to the incubator for
18 additional hrs. Antagonists or SOD-mimetics were
20 added to the cells 15 min. before NMDA exposure or 15,
30 or 60 min. following final media replacement. For
determination of Ca⁻⁻-dependence, cells were incubated in
HBSS⁻ with no added Ca⁻⁻. When kainate (KA) was used as
an agonist, 100 µM D-APV was routinely included to block
25 secondary NMDA receptor activation. Catalase (100 U/ml)
was always included when xanthine plus xanthine oxidase
(X+XO) was used as a treatment to rule out the
contribution of hydrogen peroxide.

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Neurotoxicity studies: Neurotoxicity was
determined by the measurement of LDH released in the
supernatant media as previously described (Patel et al,
Toxicol. Appl. Pharmacol. 115:124 (1991) and

5   Neurotoxicology 14:35 (1992)). LDH was measured by the
method of Vassault, Lactate dehydrogenase. In: Methods
of Enzymatic Analysis, Bergmeyer HU (ed), Verlag Chemie,
Weinheim, pp. 118-126 (1983). Additionally, cell death
was confirmed with ethidium-1 homodimer (EthD-1).

10   Briefly, the cells were washed with HBSS, loaded with
20 μM EthD-1 for 30 min., rinsed and viewed through
fluorescence optics. The number of dead cells labeled
with EthD-1 were counted in 4-6 randomly selected
fields, the numbers averaged to give an estimate of cell

15   death and the experiment was repeated 3 times.

Aconitase measurement: For the measurement of
aconitase, following treatment with agents, media was
removed and cells lysed in ice-cold 50 mM Tris/HCl, pH
7.4 containing 0.6 mM $MnCl_2$, 1 mM L-cysteine, 1 mM

20   citrate and 0.5% Triton-X 100. The aconitase activity of
cell lysates was measured spectrophotometrically by
monitoring the formation of *cis*-aconitate from
isocitrate at 240 nm in 50 mM Tris/HCl, pH 7.4,
containing 0.6 mM $MnCl_2$ and 20 mM isocitrate at 25°C

25   (Kreb and Holzach, Biochem. J. 52:527 (1952); Gardner
and Fridovich, J. Biol. Chem. 267:8757 (1992)). Inactive
aconitase was reactivated by a 30 min. incubation of
cortical cell lysates (90 μl) with 0.5 M DTT (10 μl), 20
mM Na_2S (1 μl) and 20 mM ferrous ammonium sulfate(1 μl)

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in 50 mM Tris/HCl, pH 8.0. at 37°C. Aconitase activity
in cortical cell lysates was inhibited by 0.1 mM
potassium ferricyanide or by boiling of sample.

5   *Statistical analyses:* One-way analysis of variance
(ANOVA) was used to compare three or more treatments and
Dunnet's test for comparing multiple treatment groups
with a control group. Tukey-Kramer multiple comparisons
test was used to detect differences between treatments.
Student's t-test was used for comparison of two
10   treatments.

<u>Results</u>

*Validation of aconitase activity as a marker of O_2^-
radical formation in cortical cell culture:* With the
use of agents that are known to generate O_2^- (X+XO and
15   PQ^{++}), aconitase activity was established as a valid
marker of O_2^-. The O_2^- generators selectively and
reversibly inactivated aconitase and did so in a SOD-
preventable manner.

*Aconitase inactivation produced by PQ^{++}, NMDA and KA
20   correlate with cell death:* Cortical cells were treated
with varying concentrations of KA, NMDA, and PQ^{++} for 18
hr. and the media analyzed for LDH activity. Sister
cultures were treated with varying concentrations of KA,
NMDA, or PQ^{++} for 6, 1 or 3 hr., respectively, and cell
25   lysates analyzed for aconitase activity; a shorter time
of incubation was assessed for aconitase activity

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because death of neurons leads to irreversible
inactivation of aconitase activity, presumably due to
protein degradation. Therefore, an earlier time point
was selected for measuring aconitase activity based upon
5   the time of approximating the steady state of reversible
inactivation (Fig. 34 A and B for 50 μM NMDA and 300 μM
KA, respectively).

KA treatment produced proportionate decreases in
aconitase activity and cell death as monitored by LDH
10   release (Fig. 35A).   Likewise, PQ$^{..}$ treatment produced a
proportionate decrease of aconitase activity and the
amount of LDH release (Fig. 35 B). At higher
concentrations (30-1000 μM), NMDA produced proportionate
decrease in aconitase activity and also cell death.
15   However, low concentrations (3 and 10 μM) of NMDA
produced cell death (LDH release) but no detectable
aconitase inactivation (Fig. 35 C).

*MnTBAP prevents aconitase inactivation and cell
death produced by PQ, NMDA and KA:* To determine whether
20   MnTBAP can prevent aconitase inactivation and cell death
produced by NMDA, PQ$^{..}$ and KA, cortical cells were
incubated with PQ$^{..}$, NMDA and KA in the absence and
presence of 200 μM MnTBAP. PQ$^{..}$, NMDA, and KA produced a
70 %, 40 % and 42% decrease in aconitase activity after
25   3, 1 and 6 hrs., respectively; 200 μM MnTBAP markedly
inhibited the PQ$^{..}$-, NMDA- and KA-mediated decreases of
aconitase activity (Fig. 36 A). Parallel effects were
evident on cell death as measured by LDH release.
MnTBAP (200 μM) markedly inhibited PQ$^{..}$-, NMDA- and KA-

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mediated cell death (Fig. 36 B). The effects of MnTBAP
on cell death were concentration dependent (Fig. 36 B).
MnTBAP (200 µM) produced a right-ward and down-ward
shift in the concentration-response curve for NMDA,
5   providing complete protection at low concentrations of
NMDA (Fig 37).

The possibility that MnTBAP simply prevented
activation of NMDA or AMPA receptors was examined and
eliminated.

10   To strengthen the possibility that MnTBAP decreased
aconitase inactivation and prevented cell death by its
SOD-mimetic action, the effects of the structurally
related congener, ZnTBAP, with diminished SOD activity
(ie 10-fold less) were examined. EthD-1 was used to
15   quantitate cell death in these experiments because
preliminary studies showed that ZnTBAP interfered with
the measurement of LDH (MnTBAP was found to have no
effect on LDH measurement). Concentration-response
curves for NMDA and KA demonstrated a strong correlation
20   between cell death measured using LDH release and dead
cells quantitated by EthD-1. Concentration-response
curves disclosed that MnTBAP exhibited markedly
increased neuroprotective effects in comparison to
ZnTBAP against $PQ^{..}$, NMDA and KA toxicities (Fig. 38).

25   To determine whether addition of MnTBAP after
initiation of the excitotoxic insult exerted
neuroprotective effects, 100 µM NMDA was included for 15
min. in $HBSS^-$ ($Ca^{..}-$, $Mg^{..}-$free HBSS containing 5.6 mM
glucose and supplemented with 2 mM $CaCl_2$, 1 mM $NaHCO_3$, 10
30   mM HEPES and 5 µM glycine). This acute exposure

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paradigm induced delayed neuronal death (measured 18 hr. later) and allowed determination of the temporal relationship between MnTBAP exposure and neuroprotection. The neuroprotective effects of MnTBAP

5   were assessed when it was: 1) present 15 min. prior to and during a 15 min. NMDA application (but not for the 18 hr. period after media change) (condition "pre"); 2) present 15 min. prior to and during a 15 min. NMDA application and for the ensuing 18 hr. (condition "pre +

10 post"); 3) added 15, 30 or 60 min. following a 15 min. incubation with 100 μM NMDA and left in the media for the ensuing 18 hr. Condition "pre" resulted in a 25 % reduction of LDH release measured 18 hr. later; using an identical time course of application of 100 μM D-APV in

15 condition "pre", a complete blockade of LDH release was observed. In contrast, MnTBAP incubated as in condition "pre + post" resulted in a 51% reduction of LDH release. Addition of 200 μM MnTBAP 15, 30 or 60 min. following a 15 min. exposure to 100 μM NMDA

20 resulted in a 38%, 30% and 25% reduction of LDH release respectively. In contrast, addition of 10 μM MK801 15 min. after NMDA treatment had a modest protective effect (17%) when added 15 min. after the NMDA insult. In order to investigate the possibility that NMDA-induced

25 aconitase inactivation resulted from peroxynitrite formation, the effects of nitric oxide synthase (NOS) inhibitors on NMDA-induced aconitase inactivation and cell death were examined. NMDA-induced aconitase inactivation and LDH release were unchanged by the

30 presence of 1 mM of either N^G-nitro-L-arginine methyl

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ester, N^G-nitro-L-arginine or N^G-monomethyl-L-arginine.
NOS, however, was expressed in the cortical neuronal
preparation used. Thus, peroxynitrite would not appear
to play an etiological role in NMDA-induced aconitase
5   inactivation or cell death under the conditions used.

Example XII

Learning Impairment in EC-SOD Knocked-Out Mice

EC-SOD knocked-out mice were obtained from Dr. Lena
Carlsson and Dr. Stephan Marklund, Umeå, Sweden.
10   Learning was assessed in knock-out mice and control mice
(controls were derived from (+/+) litter mates bearing
normal levels of EC-SOD) using an eight arm radical maze
in which food was placed in a well at the end of each
arm. Food was withheld from the mice for a period of
15   eight hours and then the mice were placed into the maze.
A count was made of the number of arms the mice went
down to recover food before they went down any arm for a
second time. Naive animals randomly went down between
four and five different arms of the maze before
20   beginning to repeat.
       The results presented in Figure 39 demonstrate that
when the experiment is done on 24 successive days,
control mice learn the pattern and are able to increase
the number of arms that they go down to find food
25   without error. The EC-SOD knock-out animals failed to
show any significant learning over the entire
24 sessions.

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Example XIII

Scavenging of Peroxynitrite by Mimetics

Experimental Protocols

     Cell culture: J774 macrophages were cultured in
5   DMEM medium, supplemented with L-glutamine (3.5 mmol/L)
and 10% fetal calf serum. Cells were cultured in 96-
well plates (200 μl medium/well) until confluence. To
induce the inducible isoform of nitric oxide synthase
(iNOS), fresh culture medium containing *E. coli* LPS
10   (011:B4; 10 μg/ml) alone or in combination with murine
γ-interferon (γ-IFN, 10 U/ml) was added in the presence
or absence of the NOS inhibitor, the SOD mimetic of the
combination of the two compounds for 24h. Moreover,
cells were exposed to the NO donor compounds
15   S-nitroso-N-acetyl-DL-penicillamine (SNAP) (3 mM) and
diethylamine:NO NONOate (DNO) (3 mM) for 24h or to
authentic peroxynitrite (1 mM) for 1h. Nitrite/nitrate
concentration in the medium and mitochondrial
respiration were then measured as described below.

20   *Measurement of nitrite/nitrate production:*
Nitrite/nitrate production, an indicator of NO
synthesis, was measured in the supernatant. First,
nitrate in the culture medium was reduced to nitrite by
incubation with nitrate reductase (670 mU/ml) and NADPH
25   (160 μM) at room temperature for 2h. After 2h, nitrite
concentration in the samples was measured by the Griess
reaction, by adding 100 μl of Griess reagent (1%

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sulfanilamide and 0.1% naphthylethylenediamine in 5%
phosphoric acid) to 100 μl samples of conditioned
medium. The optical density at 550 nm (OD_{550}) was
measured using a Spectramax 250 microplate reader

5   (Molecular Devices, Sunnyvale, CA). Nitrate
concentrations were calculated by comparison with OD_{550}
of standard solutions of sodium nitrate prepared in
culture medium. All measurements were corrected for the
interference of MnTBAP at this wavelength. MnTBAP (up

10   to 300 μM) did not scavenge nitrite or nitrate and did
not interfere with the activity of nitrate reductase.

     Measurement of mitochondrial respiration: Cell
respiration was assessed by the mitochondrial-dependent
reduction of 3-(4,5-dimethylthiazol-2-yl)-

15   2,5-diphenyltetrazolium bromide (MTT) to formazan.
Cells in 96-well plates were incubated at 37°C with MTT
(0.2 mg/ml) for 1 hour. Culture medium was removed by
aspiration and the cells were solubilized in DMSO
(100 μl). The extent of reduction of MTT to formazan

20   within cells was quantitated by the measurement of
OD_{550}. All measurements were corrected for the
interference of MnTBAP at this wavelength.

     Measurement of peroxynitrite-induced oxidation of
dihydrorhodamine 123: The peroxynitrite-dependent

25   oxidation of dihydrorhodamine 123 to rhodamine 123, was
measured based on the principles of the method described
by Kooy et al, Free Radical Biol. Med. 16:149 (1994).
Briefly, peroxynitrite at 5 μM was added into phosphate-

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buffered saline containing 10 μM dihydrorhodamine 123,
in the absence or presence of MnTBAP (3-100 μM). After
a 10 min incubation at 22°C, the fluorescence of
rhodamine 123 was measured using a Perkin-Elmer
5   fluorimeter (Model LS50B; Perkin-Elmer, Norwalk, CT) at
an excitation wavelength of 500 mn, emission wavelength
of 536 nm (slit widths 2.5 and 3.0 nm, respectively).

Measurement of NO-induced vascular relaxations:
New Zealand white rabbits weighing 3-4 kg were
10   anesthetized with pentobarbital (30 mg/kg). The
descending thoracic aorta was isolated, removed, cleaned
and placed in Krebs buffer (pH 7.5). Vessels were cut
into 5 mm rings and hung on stirrups connected to force
transducers. Rings were suspended in 20 ml jacketed
15   baths which were kept at 37°C and bubbled with 95% O_2
and 5% CO_2. Rings were equilibrated with 2g resting
tension for 1h before use and contracted with
phenylephrine. Saturated NO solution was prepared by
bubbling compressed NO gas through a NaOH trap and then
20   into anaerobic deionized water. Aliquots of the nitric
oxide solutions (final concentration: 75-300 nM) were
added to the rings (in the presence or absence of 100 μM
MnTBAP) and relaxant responses were recorded.

Data analysis: All values are expressed as mean ±
25   standard error of the mean of n observations, where n
represents the number of wells studied (12 wells from 2-
3 independent experiments). Data sets were examined by
analysis of variance and individual group means were

then compared with Student's unpaired t-test. A p-value
less than 0.05 was considered significant.

Results

   MnTBAP is not a scavenger of nitric oxide: MnTBAP
5   did not inhibit the relaxations in vascular rings in
response to authentic NO. Moreover, MnTBAP, at 300 μM,
did not inhibit nitrite/nitrate accumulation in the
culture medium in response to the NO donor compound SNAP
and caused a slight inhibition in response to DNO.
10   These data, and the finding that NO does not affect the
spectral changes of MnTBAP soret band, indicate that NO
does not complex with the manganese in MnTBAP.

   MnTBAP inhibits peroxynitrite-mediated oxidation:
Peroxynitrite induced significant oxidation of
15   dihydrorhodamine 123 to rhodamine 123, which was dose-
dependently inhibited by MnTBAP with 50% inhibition at
30 μM (Fig. 40). This suggests that MnTBAP, like
cysteine, urate, ascorbate and alpha-tocopherol,
inhibits the oxidations caused by peroxynitrite.

20   *MnTBAP inhibits the suppression of mitochondrial
respiration by authentic peroxynitrite in J774 cells.*
Mitochondrial respiration was profoundly inhibited by
exposure to 1 mM peroxynitrite at 1h (Fig. 41A). This
effect was partially and dose dependently prevented by
25   MnTBAP.

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MnTBAP inhibits the suppression of mitochondrial respiration by NO donors in J774 cells: Mitochondrial respiration was also inhibited by exposure to the NO donor compound SNAP (Fig. 41B) and DNO for 24h (Fig. 41C). This effect was partially and dose dependently prevented by MnTBAP.

Effects of MnTBAP on NO production and suppression of mitochondrial respiration in immunostimulated J774 macrophages: Immunostimulation of the cells by lipopolysaccharide (LPS; 10 µg/ml) alone, and, more pronouncedly, in the presence of gamma interferon (IFN; 10 U/ml), resulted in nitrite/nitrate formation and a pronounced inhibition of the mitochondrial respiration. Administration of IFN alone did not induce detectable nitrite/nitrate production and only caused a mild (<15%) suppression of respiration (n=12).

MnTBAP caused a dose-dependent inhibition of nitrite/nitrate production in cells stimulated with LPS. However, in cells immunostimulated with the combination of LPS and IFN, MnTBAP caused a less pronounced inhibition of nitrite, nitrate accumulation (300 µM). For instance, at 100 µM and 300 µM, MnTBAP caused a significant, 63 and 86% inhibition, of the LPS-induced nitrite/nitrate accumulation. When given in the combined presence of LPS and IFN, 100 µM MnTBAP only caused a 25% inhibition of nitrite/nitrate accumulation and pronounced inhibition (61%) was only observed at the highest concentration of MnTBAP tested (300 µM). L-NMA (N^G-methyl-L-arginine) caused a near-complete inhibition

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of the production of NO in cells stimulated with LPS or
LPS and IFN; and MnTBAP had no additional effect on
nitrite/nitrate formation in the presence of L-NMA. The
inhibition of nitrite/nitrate accumulation by MnTBAP in
5   LPS-stimulated macrophages diminished by more than 50%
when the agent was applied 6h after LPS, whereas in the
case of the inhibition seen with L-NMA, the extent of
inhibition seen was similar when the compound was given
together with LPS or at 6h thereafter (n=6).

10   MnTBAP caused a dose-dependent, partial restoration
of the immunostimulation-induced suppression of the
mitochondrial respiration in both LPS-treated and (less
potently) LPS and IFN-treated cells. Inhibition of NOS
with L-NMA caused a restoration of the respiration to an
15   extent comparable with that of 300 μM MnTBAP. The
combined administration of 300 μM MnTBAP and 3mM L-NMA
caused an additional restoration of the mitochondrial
respiration. In the presence of L-NMA and MnTBAP,
respiration was restored to initial levels in the LPS-
20   stimulated cells, but remained below normal in cells
stimulated with LPS and IFN.

Example XIV

Synthetic Reaction Schemes

Synthesis of 10303 (Fig. 42A)

25   1.   Methyl 2-methoxy-3-methyl benzoate ($\underline{1}$)

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To a magnetically stirred solution of 2-hydroxy-3-
methyl benzoic acid (2.22 g, 14.6 mmol), finely ground
anhydrous K_2CO_3 (8.06 g, 58.3 mmol) and acetone (150 mL)
was added $(CH_3O)_2SO_2$ (2.9 mL, 30.6 mmol). The solution
5   was stirred at room temperature for 18 hours then heated
at reflux until analysis by TLC indicated the reaction
was complete (1-2 hours). The reaction mixture was
cooled to room temperature, filtered and the excess
K_2CO_3 cake was thoroughly washed with acetone. The
10 filtrate was evaporated and the residue was redissolved
in EtOAc (100-125 mL) and triethylamine (~5-9 mL) was
added. The reaction mixture was stirred at room
temperature for 30 minutes, transferred to a separatory
funnel and washed successively with H_2O (100 mL), 2N HCl
15 (until slightly acidic), H_2O (100 mL) and brine (100
mL), then dried (Na_2SO_4) filtered and evaporated under
reduced pressure. Chromatography of the residue on
silica gel (ht of silica: 15 cm, diameter: 3 cm, eluent:
1:1 hexanes/Et_2O) provided 2.50 g (95%) of pure (1).

20 2.   Methyl 2-methoxy-4-(α,α-dibromomethyl) benzoate (2)
A magnetically stirred solution of 1 (24.43 g,
135.6 mmol), NBS (54.41 g, 305.7 mmol) and CCl_4 (1L) was
exposed to a 100 watt lamp for 6 hours. Analysis by TLC
indicated that the reaction mixture was composed of the
25 mono, di and tribrominated benzoates, wherein the di-
bromide was the major product. The reaction mixture was
quenched with H_2O (200 mL) then saturated $Na_2S_2O_3$ (500
mL) was added to destroy Br_2. The mixture was
transferred to a separatory funnel, vigorously shaken

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then separated. The organic layer was dried (Na$_2$SO$_4$),
filtered and evaporated under reduced pressure. The
crude ^{1}H NMR spectrum indicated that methyl-2-methoxy-4-
(α,α-dibromo-methyl) benzoate was the major product, and
5   was used without further purification.

3.   Methyl 4-formyl-2-methoxy-benzoate (3).
     The crude product 2 was dissolved in acetone/H$_2$O
(200 mL, 83:17) then AgNO$_3$ (47.25 g, 278.2 mmole) was
added. The flask was covered with foil to avoid
10   decomposition of the AgNO$_3$ by light. The reaction
mixture was stirred at room temperature for 2-3 hours,
then the AgBr salts were filtered off from the solution.
The filtrate was diluted with EtOAc (400 mL),
transferred to a separatory funnel, then washed with
15   saturated NaHCO$_3$ (300 mL) to extract acid away from the
aldehyde. The organic layer was washed successively
with H$_2$O (300 mL) and brine (300 mL), dried (Na$_2$SO$_4$),
filtered and evaporated under reduced pressure.
Chromatography of the residue on silica gel (ht of
20   silica: 15 cm, diameter: 5cm, eluent: 1:1 hexanes/Et$_2$O)
provided 11.92 g (42%) of pure aldehyde (3).

4.   Methyl 4-formyl-2-hydroxy benzoate (4)
     To a magnetically stirred solution of methyl 4-
formyl-2-methoxy benzoate (3) (1.82 g, 9.37 mmol) in
25   CH$_2$Cl$_2$ (35 mL) at 0°C and under N$_2$ was added dropwise,
1M BCl$_3$ (18.7 mL, 18.7 mmole). The reaction mixture was
stirred at 0°C for 10-30 minutes then quenched with H$_2$O
(50 mL). Ether (100 mL) was added to the mixture then

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transferred to a separatory funnel and layered. The
aqueous layer was extracted with ether (35 mL). The
combined organic extracts were washed with brine (100
mL), dried (Na$_2$SO$_4$), filtered, and evaporated under

5   reduced pressure. Chromatography on silica gel (ht of
silica: 12.5 cm, diameter: 5 cm, eluent: 1:1
hexanes/Et$_2$O provided 1.60 g (95%) of pure methyl 4-
formyl-2-hydroxy benzoate as an oil.

5.   Tetramethyl 2,2',2", 2'''-tetrahydroxy-4,4', 4",

10   4'''-(21H, 23H-porphine-5,10,15,20-tetrayl)tetrabenzoate
(5).
       In a foil-covered, 1L 3 neck round-bottomed flask
equipped with a reflux condenser, magnetic stirrer and a
N$_2$ inlet was added methyl 4-formyl-2-hydroxy benzoate

15   (1.01 g, 5.6 mmole), pyrrole (397 μL, 5.6 mmole) and dry
CH$_2$Cl$_2$ (560 mL). The reaction mixture was stirred at
room temperature for 10-30 minutes then BF$_3$·OEt$_2$ (69 μL,
0.56 mmole) was added. The extent of reaction was
followed by uv-vis spectrophotometry. After 1.5 hours

20   at room temperature tetrachloro-1,4-benzoquinone
(1.03 g, 4.2 mmole) was added and the reaction mixture
was heated at reflux for 3.5 hours. The reaction
mixture was allowed to cool to room temperature then the
solvent was evaporated under reduced pressure. After 2

25   chromatographic purifications on silica gel, 0.38 g
(30%) of (5) was obtained as a violet solid.

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General procedure for chromatographic purification of $\underline{5}$.

On a 1 g scale of aldehyde, the crude product was usually combined with 2-3 g silica gel to make a mixture which was then divided into 2 batches for 2 separate

5   chromatographic purifications on silica gel (ht of silica: 12-15 cm, diameter 5 cm). The first eluent used was ether 1:1 hexanes/Et_2O or 4:1 hexanes/EtOAc (depending on how porphyrin behaves on TLC) to remove hydroquinone by-products and other non-polar impurities.

10   After all non-polar impurities were removed, the eluent was changed to either CH_2Cl_2 or $CHCl_3$ to elute the porphyrin.

6.   Trimethyl 2,2',2", 2'''-tetrahydroxy-4-benzoic acid-4', 4", 4''' (porphine -5,10,15,20-tetrayl)-

15   tribenzoate manganese (III) chloride ($\underline{6}$).

A solution of $\underline{5}$ (0.38 g, 0.42 mmole) and $MnCl_2$ (0.26 g, 2.1 mmole) in DMF (25 mL) was heated at reflux for 1.5 hours, then air was bubbled for 1.5 hrs as the solution was being cooled to room temperature. The DMF

20   was evaporated under reduced pressure, then the residue was combined with silica gel (1 g) and CH_2Cl_2 (10 mL) to make a slurry. The CH_2Cl_2 was evaporated leaving a solid mixture which was loaded dry into a column packed with silica gel (ht of column: 12.5 cm, diameter: 5 cm,

25   eluent: 3% MeOH/CH_2Cl_2). The preceeding purification yielded 124 mg (32%) of pure $\underline{6}$ as a dark green solid. (mp >300°C; UV-VIS λ_{max} 467 nm (131,000); FAB-MS calculated for $C_{51}H_{34}MnN_4O_{12}$ 949, found 949)

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Synthesis of 10204 (Fig. 42B)

7.   4,4',4",4'''-(21H,23H-porphine-5,10,15,20-tetrayl)-
tetrakis(benzonitrile) (7).

     In a foil-covered 2L 3-neck round-bottomed flask
5   equipped with a reflux condenser, magnetic stirrer and a
     N$_2$ inlet was added 4-formyl benzonitrile (1.13 g, 8.6
     mmole), pyrrole (0.6 ml, 8.6 mmole) and CH$_2$Cl$_2$ (850 ml).
     The reaction mixture was stirred for 15 minutes at RT
     then BF$_3$·OEt$_2$ (105 μl, 0.85 mmole) was added. The extent
10   of reaction was followed by uv-vis spectrophotometry.
     After 2 hours, tetrachloro-1,4-benzoquinone (1.56 g,
     6.34 mmole) was added and the reaction mixture was
     heated at reflux for 2 hours. The reaction mixture was
     evaporated to a volume of 50-100 ml, then added to 2.8 g
15   of silica gel. The rest of the solvent was evaporated
     under reduced pressure to provide a solid mixture for
     chromatographic purifications. After 3 separate
     purifications, 700 mg (46%) of pure 7 was obtained as a
     powdery violet solid.

20   8.   1,1',1", 1'''-Tetra-(1H-tetrazol-5-yl)-
     4,4',4",4'''-(21H,23H-porphine-5,10,15,20-tetrayl)-
     tetrakisbenzene (8).
          A solution of 7 (0.30 g, 0.42 mmole), NaN$_3$ (0.24 g,
     3.69 mmole), NH$_4$Cl (0.18 g, 3.37 mmole) and DMF (25 ml)
25   was heated at 120°C for 3 days. Additional NaN$_3$ (0.17
     g, 2.59 mmole) and NH$_4$Cl (0.11 g, 2.05 mmole) were added
     in portions during day 2 to drive the reaction to
     completion. The DMF was evaporated under reduced

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pressure then cold H_2O (10 ml) was added. The resulting
solution was acidified with 6N HCl then CH_2Cl_2 (10 ml)
was added. The resulting precipitate was collected and
dried under vacuum to provide 0.37 g (99%) of <u>8</u> as a
greenish solid which was used without further
purification.

9.   1,1',1'',1'''-Tetra-(1H-tetrazol-5-yl)-4,4',4'',4'''-
(porphine-5,10,15,20-tetrayl)-tetrakisbenzene manganese
(III) chloride (<u>9</u>).

     A solution of <u>8</u> (0.35 g, 0.4 mmole) and $MnCl_2$
(0.25 g, 2 mmole) in DMF (20 mL) was heated at reflux
for 4-5 hours. The reaction mixture was allowed to cool
to room temperature then DMF was evaporated under
reduced pressure. The crude product was adsorbed onto
1.5 g of silica gel with 10-15 mL CH_2Cl_2. The CH_2Cl_2
was evaporated leaving a solid which was loaded dry into
a column packed with silica gel slurry (ht of silica:
12.5 cm, diameter: 5 cm, eluent: 6 $CHCl_2$/3 MeOH/1
NH_4OH). Chromatographic purification provided 0.19 g
(50%) of pure <u>9</u> as a dark green solid. (mp >320°C; UV-
VIS λ_{max} 468 nm (42,000); FAB-MS calculated for
$C_{48}H_{28}MnN_{20}$ 939, found 939)

Synthesis of 10305 (Fig. 42C)

10.   Methyl 3-methoxy-4-methyl benzoate (<u>10</u>).
     To a magnetically stirred solution of 3-hydroxy-4-
methyl benzoic acid (15.1 g, 99.2 mmole), finely ground
anhydrous K_2CO_3 (43.7 g, 317 mmole) and acetone (250 mL)

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was added (MeO)$_2$SO$_2$ (21 mL, 222 mmole). The reaction
mixture was stirred at room temperature for 24 hours
then excess K$_2$CO$_3$ was filtered off. The acetone was
removed by evaporation and the residue was redissolved
5   in EtOAc (250 mL) then Et$_3$N (15 mL) was added. The
solution was stirred at room temperature for 30 minutes,
transferred to a separatory funnel and washed
successively with H$_2$O (100 mL), 1N HCl (until slightly
acidic), NaHCO$_3$ (100 mL), H$_2$O (100 mL) and brine (100
10 mL), dried (Na$_2$SO$_4$), filtered and evaporated under
reduced pressure. Chromatography of the residue on
silica gel (ht of silica gel: 25 cm, diameter: 4 cm,
eluent: 1:1 CH$_2$Cl$_2$/hexanes) provided 16.84 g (94%) of
pure 10.

15 11. Methyl 4-formyl-3-methoxy benzoate (11).
        To a magnetically stirred solution of 10 (5.87 g,
32.6 mmol) in 1:1 CH$_3$CN/H$_2$O (250 mL) was added
consecutively CuSO$_4$·5H$_2$O (8.13 g, 32.6 mmol) and K$_2$S$_2$O$_8$
(26.4 g, 97.7 mmole). The reaction mixture was heated
20 at reflux for 50 minutes then transferred to a
separatory funnel and extracted with EtOAc (2 x 100 mL).
The combined organic extracts were dried over Na$_2$SO$_4$,
filtered and evaporated. Purification on silica gel by
gravity chromatography (ht of silica gel: 33 cm,
25 diameter: 4 cm, eluent: 5:1 hexanes/EtOAc then 4:1
hexanes/EtOAc) provided 1.47 g (23%) of pure 11.

12. Tetramethyl 3, 3', 3", 3'''-tetramethoxy-4,4',4",
4'''-(21H, 23H-porphine-5,10,15,20-tetrayl)tetrabenzoate
(12).

     In a foil-covered 2L 3 neck round-bottomed flask
5   equipped with a reflux condenser, magnetic stirrer and a
     N$_2$ inlet was added methyl 4-formyl-3-methoxy benzoate
     (11) (1.23 g, 6.3 mmole), pyrrole (448 μl, 6.3 mmole)
     and dry CH$_2$Cl$_2$ (630 mL). The reaction mixture was
     stirred for 15 minutes then BF$_3$·OEt (78 μL, 0.63 mmole)
10   was added. The reaction was monitored by uv-vis
     spectrophotometry. After 3 hours, tetrachloro-1,4-
     benzoquinone (1.17 g, 4.7 mmole) was added and the
     reaction mixture was heated at reflux for 2.5 hours.
     The reaction mixture was allowed to cool to room
15   temperature then the solvent was evaporated *in vacuo*.
     The crude product was added onto 2.6 g silica gel with
     10-15 mL, CH$_2$Cl$_2$. The CH$_2$Cl$_2$ was evaporated and the
     resulting mixture was divided into 3 equal portions for
     three separate chromotographic ·purifications. Each
20   portion was separately loaded dry into a column packed
     with silica gel (ht of silica gel: 15 cm, diameter:
     5 cm, eluent: 1:1 hexanes/Et$_2$O then CH$_2$Cl$_2$). The
     combined product 0.461 g (30%) of pure 12 was obtained
     as a mixture of rotational isomers.

13. Tetramethyl 3,3',3",3'''-tetramethoxy-4,4',4",4'''-
(porphine-5,10,15,20-tetrayl)-tetrabenzoate manganese
(III) chloride (13)

5   A solution of 12 (0.32 g, 0.33 mmole) and MnCl$_2$
(0.20 g, 1.56 mmole) in DMF (32 mL) was heated at reflux
for 6.5 hours. Air was then bubbled into the solution
for 1.25 hours as the reaction mixture cooled to room
temperature. The DMF was evaporated off and the crude
mixture was adsorbed onto 1.5 g silica gel with 10-15 mL

10   CH$_2$Cl$_2$. The CH$_2$Cl$_2$ was evaporated and the resulting
solid mixture was loaded dry into a column packed with
silica gel (ht of silica: 12.5 cm, diameter: 5 cm,
eluent: 5% MeOH in CH$_2$Cl$_2$). Chromatographic
purification provided 294 mg (84%) of pure 13 as a

15   green-black solid. (mp >300°C; UV-VIS λ_{max} 467 nm
(145,000); FAB-MS calculated for C$_{56}$H$_{44}$MnN$_4$O$_{12}$ 1019, found
1019).

14. 3,3',3",3'''-Tetramethoxy-4,4',4",4'''-(porphine-
5,10,15,20-tetrayl)tetrakis(benzoic acid) (14).

20   A magnetically stirred solution of manganated
tetrabenzoate 13 in 10 mL Claisen's case (KOH/MeOH/H$_2$O)
was heated at reflux for 40-60 minutes. The reaction
was cooled to room temperature then to 0°C. The cold
solution was then acidified with 6N HCl then MeOH was

25   evaporated under reduced pressure. The solids were
filtered away from the acidic solution and was washed
thoroughly with cold water. The solids were collected
and dried under vacuum at 80°C overnight provide 96 mg
(87%) of pure 14 as a dark green solid (mp >300°C; uv-

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vis; λ_{max} 467 nm (150,000); FAB-MS calculated for
$C_{52}H_{36}MnN_4O_{12}$ 963, found 963).

<u>Synthesis of 10109 (Fig. 42D)</u>

15. 4,4',4",4'''-(Porphine-5,10,15,20-tetrayl)-
tetrakis(benzoylchloride) manganese (III) chloride (<u>15</u>)
        Mn TBAP (500 mg, 0.57 mmole) was suspended in dry
benzene (125 ml) in a 3-neck round bottom flask equipped
with a reflux condenser and a N_2 inlet. Thionylchloride
(45 ml; 617 mmol) was added to the mixture and the
reaction mixture was heated at reflux for 22 hours. The
benzene and the unreacted $SOCl_2$ was removed by
distillation. The residue was washed with dry benzene
(50 ml) and evaporated on rotary evaporator to dryness.
This washing and evaporating was repeated twice. The
dark green residue was dried under high vacuum to yield
0.54 g (100%).

16. Tetra(N,N-dimethyl) 4,4',4",4'''-(porphine-
5,10,15,20-tetrayl)-tetrakis(benzamide) manganese (III)
chloride (<u>16</u>).
        To compound <u>15</u> (340 mg, 0.36 mmole) in THF (20 mL)
was added dimethylamine in THF solution (10 ml, 2M
solution). The reaction mixture was refluxed for 6
hours. TLC analysis (eluent: 1 methanol:3 chloroform)
showed complete conversion of the acid-chloride to the
product. After cooling to room temperature, deionized
water (100 ml) was added to the reaction mixture and the
solid was filtered off. The crude solid was purified by

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column chromatography on silica gel (methylene
chloride:methanol = 4:1 eluent). The combined fractions
(Rf = 0.75 in CH_2Cl_2:MeOH = 4:1) were evaporated, and
dried under high vacuum, to provide the product as a
green solid 0.11 g (31%). (UV-VIS λ_{max} 467 nm (56,100);
FAB-MS calculated for $C_{56}H_{45}MnN_8O_4$ 952, found 952.)

Synthesis of 10402, 10602, 11001, 11002, 11003 and 11103

Insofar as compounds 10402, 10602, 11001, 11002,
11003, and 11103 are concerned, synthesis can be carried
out essentially, as described in Example XIV sections 5
and 6; the following starting material substitutions are
required: 4-hydroxybenzaldehyde for methyl 4-formyl-2-
hydroxy benzoate in the case of 10402, 4-hydroxy-3-
nitrobenzaldehyde for methyl 4-formyl-2-hydroxy benzoate
in the case of 10602, indole-3-carboxaldehyde for methyl
4-formyl-2-hydroxy benzoate in the case of 11001, 4-
quinolinecarboxaldehyde for methyl 4-formyl-2-hydroxy
benzoate in the case of 11002, 3-quinolinecarboxaldehyde
for methyl 4-formyl-2-hydroxy benzoate in the case of
11003; and 3-fluoro-4-methoxybenzaldehyde for methyl
4-formyl-2-hydroxy benzoate in the case of 11103.

Synthesis of 10207 and 10208

Acetonitrile is reacted with hydroxylamine to
prepare the amide oxime. An excess of the amide oxime
is allowed to react with the product described in
Example XIV, section 15, to provide a mixture of 10207

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and 10208. Chromatographic purification of the reaction
mixture provides pure 10207 and 10208.

Synthesis of 10209

Commercially available 4-methylbenzoic acid is
5   transformed into its acid chloride by reaction with
oxalyl chloride or thionyl chloride. The resulting acid
chloride is heated in the presence of cuprous cyanide to
provide the α-ketonitrile derivative. The α-ketonitrile
is hydrolyzed with anhydrous methanol saturated with
10   anhydrous hydrogen chloride gas to give the
α-ketonitrile ester derivative. The aromatic methyl
group is efficiently oxidized to the aldehyde oxidation
state by first reaction with N-bromosuccinimide and
subsequent hydrolysis with silver nitrate. Synthesis of
15   10209 can be carried out essentially as described in
Example XIV, sections 5 and 6; the substitution of
starting material of methyl 4-formyl-2-hydroxy benzoate
with the aldehyde described hereinabove being required.

*   *   *

20   All documents cited above are hereby incorporated
in their entirety by reference.
One skilled in the art will appreciate from a
reading of this disclosure that various changes in form
and detail can be made without departing from the true
25   scope of the invention. In addition to the compounds
described herein, compounds disclosed in the following

135

references can also be used as oxidant scavengers
consistent with the invention: USP 5,227,405; Nagele et
al, Biochem. Pharmacol. (UK) 47:555-562 (1994); Baudry
et al, Biochem. Biophys. Res. Comm. 192:964-968 (1993);
5   Duran et al, Cancer Lett (IRE) 69:167-172 (1993); Itami
et al, Biochem. Biophys. Res. Comm. 197:536-541 (1993);
Kitajima et al, Inorg. Chem. 32:1879-1880 (1993); Riley
et al, Free Radical Biol. & Med. 15:514 (1993); Weiss et
al, J. Biol Chem. (US) 268:23049-23054 (1993); Foye,
10   Ann. Pharmacother. 26:1144-1147 (1992); Haseloff et al,
J. Biolumin. Chemilumin. (UK) 7:171-175 (1992);
Pelletier, J., Biochem. Pharmacol. 43:1061-1066 (1992);
Yaping et al, J. Free Radic. BiolMed 13:533-541 (1992);
Schechinger et al, Biol. Met. 1:112 (1988); Linss et al,
15   Inorg. Chim. Acta 125:117 (1986); and Weser et al, Adv.
Exp. Med. Biol. 264:51 (1990).

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Footnote to Table IX

[1]Porphine carbon numbers refer to the methine bridge carbons (5, 10, 15, 20) at which each R group is attached, or to the pyrrole carbon (2, 4, 7, 8, 12, 13, 17 or 18), at which P is attached. P is hydrogen unless otherwise defined.

[2]SOD activity determined as described by McCord and Fridovich, J. Biol. Chem. 244:6049 (1969).

[3]Catalase activity determined as described by Del Rio et al, Anal. Biochem. 80:409 (1977). Metalloporphyrins were assayed at various concentrations in the presence of 1 mM hydrogen peroxide for catalase-like activity. A Clark electrode was used to measure the formation of oxygen from the breakdown of hydrogen peroxide over a period of 2 minutes.

[4]Rates of growth of cultures of null SOD *E. coli* (JI132 strain) were followed turbidimetrically at 700 nm to minimize interferences from the absorbance of test compounds. Growth medium contained 0.2% glucose, 0.2% casamino acids, 30 mg/L thiamine, 30 mg/L pantothenic acid, and M9 salts in water, pH adjusted to 7.0. Test compounds were filter-sterilized before adding to media. (Faulkner et al, J. Biol. Chem. 269:23471 (1994)).

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[5]Human umbilicord vein endothelial cells (HUVEC) (ATCC #CRL-1730) (Moldow et al, Methods in Enzymology 105:378-385 (1984)) were grown in Ham's F-12K medium supplemented with 10% fetal bovine serum, 0.1 mg heparin

5   and 0.03 mg/ml endothelial cell growth factor in T-75 culture flasks in a humidified atmosphere containing 5% carbon dioxide and 95% air. Cells were plated at ~3.5 x 10^4 cells/well in 24-well plates. Experiments were performed 48 hours later. Medium was changed to

10 EMEM without serum supplement but including heparin and endothelial cell growth factor at the start of each study. Stock solutions of D-aminoacid oxidase and metalloporphyrins were diluted into (EMEM) and passed through a 0.2cc filter before use. HUVEC cells were

15 labelled with Cr^{51} by incubating them with EMEM containing radiolabeled sodium chromate for 4 hrs at an isotope concentration of $20\mu Ci/10^6$ cells. Unincorporated Cr^{51} was removed by repeated washes in EMEM. Injury was initiated by incubation of endothelial

20 cells for 5 hours with a hydrogen peroxide generating system consisting of D-alanine (1 mM) and 40 mU/ml of D-aminoacid oxidase in EMEM. D-amino acid oxidase is found in PMNs (polymorphonuclear leucocytes) and is thought to be part of the oxidative burst which is a key

25 part of the PMN mediated inflammatory response (Robinson et al, J. Cell Biology, 77:59 (1978); Cline et al, Microbiology 62:756 (1969); De Chatelet J. Reticul. Soc. 24:73 (1978)). Supernatant from each well was collected at the end of the incubation period, cells were washed

30 with PBS and then lysed with 0.4 N sodium hydroxide.

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
Radioactivity in both supernatants and cell lysates were
measured separately in a gamma counter. A set of wells
in each plate were used to measure basal release of Cr^{51}
from HUVEC incubated in EMEM alone. Cr^{51} release was
5   expressed as a percent of radioactivity in supernatant
to total radioactivity [(Cr^{51} in supernatant+ CR^{51} in
lysate) x 100].

ACTIVITY OF COMPOUNDS

TABLE IX

Compound (metal complexes)	Porphyrin Carbon number(s)	R1′ (or R1′, or P1′)	R2 (or R2′)	R3 (or R1′)	side group class	SOD[3] µg/USOD	Catalase[3] min^{-1}	Null SOD[4] E. Coli bioassay	H$_2$O$_2$ HUVEC[5]
(Mn) 10101	5,10,15,20	⟨phenyl⟩–O=C–NH–	–(CH$_2$)$_2$–	–NH$_2$	cationic	213	3.74	+	+
(Mn) 10104	5,10,15,20	⟨phenyl⟩–O=C–NH–	–(CH$_2$)$_2$–	–N$^+$(CH$_3$)$_3$	cationic	349	N.D.	N.D.	N.D.
10105 (Mn)	5,10,15	⟨phenyl⟩–O=C–NH–	–(CH$_2$)$_2$–	–N$^+$(CH$_3$)$_3$	cationic	445	1.11	–	–
	20	⟨phenyl⟩	Bond	–COOH					
	5,20	⟨phenyl⟩	Bond	–COOH					
10106 (Mn)	10,15	⟨phenyl⟩–O=C–NH–	–(CH$_2$)$_2$–	–N$^+$(CH$_3$)$_3$	cationic	140	0.53	+	–

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Compound (metal complexes)	Porphyrin Carbon number(s)	R1 (or R1, P1)	R2 (or R2)	R3 (or R3)	side group class	SOD[5] μg/USOD	Catalase[4] min⁻¹	Null SOD[4] E. Coli bioassay	H₂O₂ HUVEC[5]
10107 (Mn)	5,15	⬡-C(O)-NH-	-(CH₂)₂-	-N⁺(CH₃)₃	cationic	172	1.69	-	+
	10,20	⬡-	Bond	-COOH					
10108 (Mn)	5	⬡-C(O)-NH-	-(CH₂)₂-	-N⁺(CH₃)₃	cationic	375	0.88	+	-
	10,15,20	⬡-	Bond	-COOH					
(Mn) 10109	5,10,15,20	⬡-	Bond	-N(CH₃)₂	cationic	470	0.59	-	+
(Mn) 10201	5,10,15,20	⬡-	Bond	-COOH	anionic	33	0.8	+	+
(Mn) 10202	5,10,15,20	⬡-C₆O₂H₄	Bond	-H	anionic	15	1.5	-	+
(Mn) 10203	5,10,15,20	⬡-	Bond	-SO₃H	anionic	inactive	0.9	+	+
(Mn) 10204	5,10,15,20	⬡-	Bond	[imidazole]	anionic	1.3	0.33	-	-

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Compound (metal complexes)	Porphyrin Carbon number(s)	R1 (or R1') (or P1)	R2 (or R2')	R3 (or R3')	side group class	Biochemistry SOD[2] µg/USOD	Biochemistry Catalase[1] min^-1	In Vitro Null SOD[4] E. Coli bioassay	In Vitro H_2O_2 HUVEC[5]
(Mn) 10205	5,10,15,20	phenyl	Bond	oxazoline–CH_3	anionic	28	1.8	–	–
(Mn) 10206	5,10,15,20	phenyl	Bond	oxazoline–CH_3	anionic	447	1.9	–	–
10207 (Mn)	5,10,20	phenyl	Bond	oxazoline–CH_3	anionic	inactive	inactive	–	–
	15	phenyl	Bond	-COOH	anionic				–
	5,15	phenyl	Bond	-COOH	anionic				–
10208 (Mn)	10,20 *	phenyl	Bond	oxazoline–CH_3	anionic	201	inactive	N.D.	–
(Mn) 10209	5,10,15,20	aryl–C(O)…C(O)–	Bond	$-OCH_3$	anionic	inactive	0.1	–	–
(Mn) 10301	5,10,15,20	aryl–CO_2CH_3	Bond	-OH	chelator	314	0.85	+	–
(Mn) 10302	5,10,15,20	aryl–CO_2CH_3	Bond	$-OCH_3$	chelator	37	2.9	–	–

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Compound (metal complexes)	Porphyrin Carbon number(s)	R1 (or R₁) (or P₁)	R2 (or R₂)	R3 (or R₃)	side group class	Biochemistry SOD² µg/USOD	Catalase³ min⁻¹	In Vitro Null SOD³ E. Coli bioassay	H₂O₂ HUVEC⁵
10303 (Mn)	5	(structure)	Bond	-OH	chelator	78	1.4	+	+
10304 (Mn)	10,15,20	(structure)	Bond	-OH	chelator	ppt	ppt	-	-
10305 (Mn)	5,10,15,20	(structure)	Bond	-OH	chelator	inactive	2.2	+	+
10306 (Mn)	5,10,15,20	(structure)	Bond	-OCH₃	chelator	50	9.9	+	-
10401 (Mn)	5,10,15,20	(structure)	Bond	-OCH₃	anti-oxidant	insol	insol	N.D.	N.D.
10402 (Mn)	5,10,15,20	(structure)	Bond	-OH	anti-oxidant	268	inactive	-	-
10601 (Mn)	5,10,15,20	(structure)	Bond	-NO₂	nitro	18	0.5	+	-
10602 (Mn)	5,10,15,20	(structure)	Bond	-NO₂	nitro	74	0.1	+	+
10701 (Mn)	5,10,15,20	(structure)	Bond	-H	un-substit	96	2.1	-	-

143

Compound (metal complexes)	Porphyrin Carbon number(s)	R1 (or R'1)(or P1)	R2 (or R'2)	R3 (or R'3)	side group class	Biochemistry SOD³ μg/USOD	Biochemistry Catalase¹ min⁻¹	In Vitro Null SOD² E. Coli bioassay	In Vitro H₂O₂ HUVEC³
(Mn) 10901	5,10,15,20	(phenyl ring)	Bond	–CN	cyano	169	3.2	–	–
(Mn) 11001	5,10,15,20	Bond	Bond	(heterocyclic structure)	hetero-cyclic	404	0.9	N.D.	–
(Mn) 11002	5,10,15,20	Bond	Bond	(quinoline structure)	hetero-cyclic	22	3.3	N.D.	–
(Mn) 11003	5,10,15,20	Bond	Bond	(heterocyclic structure)	hetero-cyclic	106	1.02	N.D.	+
11101 (Cu)	5,10,15,20 / 2,3,7,8,12,13,17,18	(phenyl ring) / –Br	Bond	–CH₃	halo-genated	inactive	inactive	N.D.	N.D.
(Mn) 11102	5,10,15,20 / 2,3,7,8,12,13,17,18	(phenyl ring) / –Br	Bond	–CH₃	halo-genated	0.6	4.6	N.D.	N.D.
(Mn) 11103	5,10,15,20	(phenyl–COOH ring)	Bond	–F	halo-genated	N.D.	2.4	N.D.	PPT

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WHAT IS CLAIMED IS:

1.   An oxidant scavenger comprising a nitrogen-containing macrocyclic moiety and a cell surface or extracellular matrix targeting moiety, or a pharmaceutically acceptable salt thereof.

2.   The scavenger according to claim 1 wherein said macrocyclic moiety is a porphyrin.

3.   The scavenger according to claim 1 wherein said porphyrin is tetrakis (4-benzoic acid) porphyrin.

4.   An oxidant scavenger of the formula:

or a pharmaceutically acceptable salt thereof, wherein:

R$_1$ is a bond, $-\langle H \rangle$, $-\langle \bigcirc \rangle$, $-\langle \bigcirc \rangle N^{\pm}$, $\langle \bigcirc \rangle^{NO_2}$, $-\langle \bigcirc \rangle^{SO_3H}$, $-\langle \bigcirc \rangle^{SO_3^-}$, $-\langle \bigcirc \rangle^{X}$, or $-\langle \bigcirc \rangle^{Y}$ wherein X is a halogen and Y is an alkyl group and wherein $-\langle \bigcirc \rangle$ indicates bonding to R$_2$ at any position and $-\langle \bigcirc \rangle$

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indicates bonding to R_2 and the substituent at any

position; and

    R_2 is a bond, $-(CY'_2)_n^-$, $-(CY'_2-CY'=CY')_n^-$, $-(CY'_2-$
$CY'_2-CH=CH)_n^-$, $-(CY'=CY')_n^-$, or $-(CY'_2-\overset{O}{\overset{\parallel}{C}})_n^-$, wherein Y'
is hydrogen or an alkyl group and wherein n is 1 to 8;
and

    R_3 is $-Y''$, $-OH$, $-NH_2$, $-N^+(Y'')_3$, $-COOH$, $-COO^-$,
$-SO_3H$, $-SO_3^-$, $-C-PO_3H_2$ or $-C-PO_3H^-$, wherein Y'' is an
alkyl group,

    wherein, when R_1 is and R_2 is a bond, R_3 is
not Y'', and

    wherein, when R_1 is and R_2 is a bond, R_3 is
not $-Y''$, $-N^+(Y'')_3$, or COOH.

    5.   The scavenger according to claim 4 wherein at
least one of said R_1's, R_2's, or R_3's is different than
the other of said R_1's, R_2's, or R_3's, respectively.

    6.   The scavenger according to claim 1 or claim 4
wherein said scavenger is complexed with a metal
selected from the group consisting of manganese, copper
and iron.

    7.   The scavenger according to claim 1 wherein
said targeting moiety is a sugar or is selected from the
group consisting of:
    $(NH-CH_2-CH_2-NH)_n-H$ where n = 1-6;

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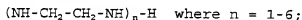
NH_2 - His Arg His His Pro Arg Glu Met Lys Lys Arg
Val Glu Asp Leu - COOH;

NH_2 - Arg Glu His Ser Glu Arg Lys Lys Arg Arg Arg
Glu Ser Glu Cys Lys Ala Ala - COOH;

NH_2 - Arg Glu His Ser Glu Arg Lys Lys Arg Arg Arg
Glu - COOH;

NH_2 - Arg Glu His Ser Glu Arg Lys Lys Arg Arg Arg
Ala - COOH;

NH_2 - Arg Glu His Ser Glu Arg Lys Lys Arg Arg Arg
Ala Ser Glu Cys Lys Ala Ala - COOH;

NH_2 - Arg Glu His Ser Glu Arg Lys Lys Arg Arg Arg
Glu Ser Glu Ala Lys Ala Ala - COOH;

NH_2 - Arg Glu His Ser Glu Arg Lys Lys Arg Arg Arg
Glu Ser Glu Cys Ala Ala Ala - COOH;

NH_2 - Arg Glu His Ser Glu Arg Lys Lys Arg Arg Arg
Ala Ser Ala Cys Lys Ala Ala - COOH;

NH_2 - Arg Glu His Ser Glu Arg Lys Lys Arg Arg Arg
Ala Ser Glu Cys Ala Ala Ala - COOH;

NH_2 - Arg Glu His Ser Glu Arg Lys Lys Gly Arg Arg
Ala Ser Glu Cys Ala Ala Ala - COOH; and

$(Arg)_n$, $(Lys)_n$, $(Arg)_n$ $(Lys)_n$, $(Arg\ Lys)_n$ $(Lys$
$Arg)_n$, and $(Lys\ Lys\ Arg\ Arg)_n$, wherein n is 1 to 12.

8.   A method of protecting cells from oxidant-
induced toxicity comprising contacting said cells with a
non-toxic amount of said scavenger according to claim 1
or a scavenger of the formula,

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or a pharmaceutically acceptable salt thereof,
wherein:

R_1 is a bond, ⟨H⟩ , ⟨◯⟩ , ⟨◯⟩-N±, ⟨◯⟩-NO$_2$,

⟨◯⟩-SO$_2$H , ⟨◯⟩-SO$_3^-$, ⟨◯⟩-X , or ⟨◯⟩-Y wherein X is a

halogen and Y is an alkyl group and wherein ⟨◯⟩

indicates bonding to R_2 at any position and ⟨◯⟩

indicates bonding to R_2 and the substituent at any

position; and

R_2 is a bond, $-(CY'_2)_n^-$, $-(CY'_2-CY'=CY')_n^-$,
$-(CY'_2-CY'_2-CH=CH)_n^-$, $-(CY'=CY')_n^-$, or $-(CY'_2-\overset{O}{\underset{\|}{C}})_n^-$,
wherein Y' is hydrogen or an alkyl group and wherein n
is 1 to 8; and

R_3 is $-Y''$, $-OH$, $-NH_2$, $-N^+(Y'')_3$, $-COOH$, $-COO^-$,
$-SO_3H$, $-SO_3^-$, $-C-PO_3H_2$ or $-C-PO_3H^-$, wherein Y'' is an
alkyl group,

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optionally complexed with a metal selected from the
group consisting of manganese, copper and iron,
sufficient to effect said protection.

9.   The method according to claim 8 wherein said
scavenger is complexed with a metal selected from the
group consisting of manganese, copper or iron.

10.   The method according to claim 8 wherein said
cells are mammalian cells or plant cells.

11.   A method of inhibiting damage due to oxidation
of a substance comprising contacting to said substance
with a non-toxic amount of said scavenger according to
claim 1 or a scavenger of the formula,


or a pharmaceutically acceptable salt thereof,
wherein:

R$_1$ is a bond, $-\langle H \rangle$, $-\langle \bigcirc \rangle$, $-\langle \bigcirc \rangle N^{\pm}$, $\langle \bigcirc \rangle NO_2$,

$-\langle \bigcirc \rangle SO_3H$, $-\langle \bigcirc \rangle SO_3^-$, $-\langle \bigcirc \rangle X$, or $\langle \bigcirc \rangle Y$ wherein X is a

halogen and Y is an alkyl group and wherein $-\langle \bigcirc \rangle$

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indicates bonding to R_2 at any position and ⊘

indicates bonding to R_2 and the substituent at any

position; and

R_2 is a bond, $-(CY'_2)_n^-$, $-(CY'_2-CY'=CY')_n^-$,
$-(CY'_2-CY'_2-CH=CH)_n^-$, $-(CY'=CY')_n^-$, or $-(CY'_2-\overset{O}{\overset{\|}{C}})_n^-$,

wherein Y' is hydrogen or an alkyl group and wherein n

is 1 to 8; and

R_3 is $-Y''$, $-OH$, $-NH_2$, $-N^+(Y'')_3$, $-COOH$, $-COO^-$,

$-SO_3H$, $-SO_3^-$, $-C-PO_3H_2$ or $-C-PO_3H^-$, wherein Y'' is an

alkyl group,

optionally complexed with a metal selected from the

group consisting of manganese, copper and iron,

sufficient to effect said inhibition.

12. The method according to claim 11 wherein said

mimetic is complexed with a metal selected from the

group consisting of manganese, copper or iron.

13. A method of treating a pathological condition

of a patient resulting from degradation of NO· or a

biologically active form thereof comprising

administering to said patient an effective amount of a

compound having the activity of a catalytic antioxidant

under conditions such that said treatment is effected.

14. The method according to claim 13 wherein said

biologically active form of NO· is a nitroso, thiol,

nitrosyl, NO^+ or NO^- group.

15. A method of treating a pathological condition
of a patient resulting from peroxynitrite accumulation
comprising administering to said patient an effective
amount of a compound having the activity of a catalytic
antioxidant under conditions such that said treatment is
effected.

16. The method according to claim 13 or 15 wherein
said compound is a porphyrin ring-containing compound.

17. A method of treating an inflammatory condition
in a patient in need of such treatment comprising
administering to said patient an effective amount of a
mimetic of SOD, EC-SOD, catalase or peroxidase under
conditions such that said treatment is effected.

18. The method according to claim 17 wherein a GAG
binding moiety is attached to said mimetic.

19. A method of treating a disorder resulting from
aberrant smooth muscle function in a patient in need of
such treatment comprising administering to said patient
an effective amount of a mimetic of SOD, EC-SOD,
catalase or peroxidase under conditions such that said
treatment is effected.

20. The method according to claim 19 wherein a GAG
binding moiety is attached to said mimetic.

21. The method acording to claim 17 or 19 wherein said mimetic is a porphyrin ring-containing mimetic.

22. A pharmaceutical composition comprising the scavenger according to claim 1, 4, 28 or 41 and a pharmaceutically acceptable carrier.

23. A kit comprising the scavenger according to claim 1, 4, 28 or 41 disposed within a container means.

24. An isolated EC-SOD gene having the sequence shown in Figure 24, or portion thereof at least 18 nucleotides in length.

25. The gene according to claim 24 wherein said portion corresponds to a noncoding region of said gene.

26. A method of inhibiting xanthine oxidase activity of a cell or tissue comprising contacting said cell or tissue with an amount of said scavenger according to claim 1 or a mimetic of the formula,

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or a pharmaceutically acceptable salt thereof,

wherein:

R_1 is a bond, $\boxed{H}$, $\bigcirc$, $\bigcirc N^{\underline{\cdot}}$, $\bigcirc^{NO_2}$,

$\bigcirc^{SO_3H}$, $\bigcirc^{SO_3^-}$, $\bigcirc^{X}$, or $\bigcirc^{Y}$ wherein X is a

halogen and Y is an alkyl group and wherein $\bigcirc$

indicates bonding to R_2 at any position and $\bigcirc$

indicates bonding to R_2 and the substituent at any

position; and

R_2 is a bond, $-(CY'_2)_n^-$, $-(CY'_2-CY'=CH)_n^-$,

$-(CY'_2-CY'_2-CH=CH)_n^-$, $-(CY'=CY')_n^-$, or $-(CY'_2-\overset{\text{H}}{\underset{\text{O}}{C}})_n^-$,

wherein Y' is hydrogen or an alkyl group and wherein n

is 1 to 8; and

R_3 is $-Y''$, $-OH$, $-NH_2$, $-N^+(Y'')_3$, $-COOH$, $-COO^-$,

$-SO_3H$, $-SO_3^-$, $-C-PO_3H_2$ or $-C-PO_3H^-$, wherein Y'' is an

alkyl group,

optionally complexed with a metal selected from the

group consisting of manganese, copper and iron,

sufficient to effect said inhibition.

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27. The method according to claim 26 wherein said
pathological condition is of the lungs of said patient
and said administration is to the airways of said
patient and said compound has the tissue specificity of
extracellular superoxide dismutase.

28. An oxidant scavenger of the formula:

or a pharmaceutically acceptable salt thereof, or metal
complex thereof wherein said metal is selected from the
group consisting of manganese, copper and iron,
wherein:

each R_1' is independently a bond, , ,

, , , , , or

, wherein Y" is an alkyl group, and wherein
indicates bonding to R_2' at any position and
indicates bonding to R_2' and the R_1' phenyl substituent
at any position;

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each R_2' is independently a bond, or $-(CH_2)_n-$
wherein n is 1-4,

each R_3' is independently -Y", -Y'", -H, -OH, -OY",
$-NO_2$, -CN, $-NH_2$, -COOH, -COY", $-COO^-$, or a heterocyclic
group, wherein Y" is as defined above and Y'" is a
primary, secondary, tertiary or quaternary amine,

wherein when R_1' is ⟨phenyl⟩$-\overset{O}{\overset{\|}{C}}-\overset{O}{\overset{\|}{C}}-$, R_3' is not COOH, COY"
or COO^-,

wherein when R_1' is ⟨phenyl⟩$-\overset{O}{\overset{\|}{C}}-NH-$, ⟨phenyl⟩$-\overset{O}{\overset{\|}{C}}-\overset{O}{\overset{\|}{C}}-$, or ⟨phenyl⟩$-\overset{O}{\overset{\|}{C}}-$,
R_3' is not $-NO_2$, and

wherein $-R_1'-R_2'-R_3'$, collectively, are not -H,

⟨phenyl⟩$-CH_3$, ⟨phenyl⟩$-COOH$, ⟨phenyl⟩ , ⟨phenyl⟩$-N^+(CH_3)_3$, ⟨phenyl⟩$-COO^-$ or ⟨phenyl⟩$-CO_2CH_3$.

29. The scavenger according to claim 28 wherein
Y'''' is a secondary, tertiary or quaternary amine and
each hydrogen replacement group on the amine nitrogen is
a C_1-C_4 alkyl group.

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30. The scavenger according to claim 28 wherein said scavenger has the structure
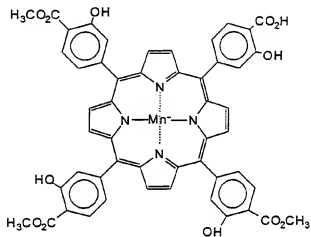
31. The scavenger according to claim 28 wherein said scavenger has the structure

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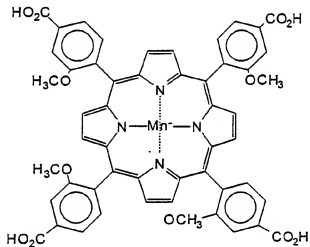
32. The scavenger according to claim 28 wherein said scavenger has the structure

33. The scavenger according to claim 28 wherein said scavenger has the structure

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34.   A method of protecting cells from oxidant-
induced toxicity comprising contacting said cells with a
non-toxic amount of a scavenger of the formula,

or a pharmaceutically acceptable salt thereof, or metal
complex thereof wherein said metal is selected from the
group consisting of manganese, copper and iron,
wherein:

each R_1' is independently a bond, , ,

, , , , , or

, wherein Y" is an alkyl group, and wherein

indicates bonding to R_2' at any position and

indicates bonding to R_2' and the R_1' phenyl substituent

at any position;

each R_2' is independently a bond, or $-(CH_2)_n-$
wherein n is 1-4,

each R_3' is independently -Y", -Y'", -H, -OH, -OY",
$-NO_2$, -CN, $-NH_2$, -COOH, -COY", $-COO^-$, or a heterocyclic

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group, wherein Y" is as defined above and Y'" is a
primary, secondary, tertiary or quaternary amine,

    where Y" is as defined above,

    wherein when R_1' is $\begin{smallmatrix}O&O\\\|&\|\\C-C-\end{smallmatrix}$, R_3' is not COOH, COY"
or COO⁻,

    wherein when R_1' is $\begin{smallmatrix}O\\\|\\C-NH-\end{smallmatrix}$, $\begin{smallmatrix}O&O\\\|&\|\\C-C-\end{smallmatrix}$, or $\begin{smallmatrix}O\\\|\\C-\end{smallmatrix}$,
R_3' is not $-NO_2$, and

    wherein $-R_1'-R_2'-R_3'$, collectively, are not -H,

    sufficient to effect said protection.

    35. The method according to claim 34 wherein said
cells are mammalian cells or plant cells.

    36. A method of inhibiting damage due to oxidation
of a substance comprising contacting to said substance
with a non-toxic amount of a scavenger of the formula,

or a pharmaceutically acceptable salt thereof, or metal
complex thereof wherein said metal is selected from the
group consisting of manganese, copper and iron,

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wherein:

each R_1' is independently a bond, ![phenyl with CO₂Y″] , ![phenyl with OH] ,

![cyclohexyl] , ![phenyl with Y″] , ![phenyl with CO₂H] , ![phenyl with C(=O)-NH-] , ![phenyl with C(=O)C(=O)-] , ![phenyl with C(=O)-] or

![phenyl with C(Y″)₃ C(Y″)₃] , wherein Y″ is an alkyl group, and wherein ![phenyl]

indicates bonding to R_2' at any position and ![phenyl]

indicates bonding to R_2' and the R_1' phenyl substituent
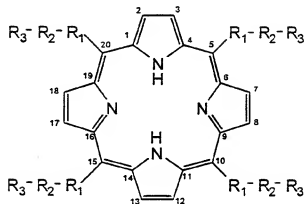
at any position;

each R_2' is independently a bond, or $-(CH_2)_n-$ wherein n is 1-4,

each R_3' is independently -Y″, -Y′″, -H, -OH, -OY″, -NO$_2$, -CN, -NH$_2$, -COOH, -COY″, -COO⁻, or a heterocyclic group, wherein Y″ is as defined above and Y′″ is a primary, secondary, tertiary or quaternary amine,

wherein when R_1' is ![phenyl with C(=O)C(=O)-] , R_3' is not COOH, COY″ or COO⁻,

wherein when R_1' is ![phenyl with C(=O)-NH-] , ![phenyl with C(=O)C(=O)-] , or ![phenyl with C(=O)-] , R_3' is not -NO$_2$, and wherein $-R_1'-R_2'-R_3'$, collectively, are not -H, sufficient to effect said protection.

37. A method of inhibiting xanthine oxidase activity of a cell or tissue comprising contacting said cell or tissue with an amount of scavenger of the formula,

or a pharmaceutically acceptable salt thereof, or metal complex thereof wherein said metal is selected from the group consisting of manganese, copper and iron, wherein:

each R_1' is independently a bond, ![CO2Y"], ![OH],

![phenyl], ![Y"], ![CO2H], ![C-NH-], ![C-C-], ![C.] or

![C(Y")3 C(Y")3], wherein Y" is an alkyl group, and wherein ![phenyl]

indicates bonding to R_2' at any position and ![phenyl]

indicates bonding to R_2' and the R_1' phenyl substituent

at any position;

each R_2' is independently a bond, or $-(CH_2)_n-$ wherein n is 1-4,

each R_3' is independently $-Y"$, $-Y'"$, $-H$, $-OH$, $-OY"$, $-NO_2$, $-CN$, $-NH_2$, $-COOH$, $-COY"$, $-COO^-$, or a heterocyclic group, wherein Y" is as defined above and Y'" is a primary, secondary, tertiary or quaternary amine,

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wherein when R_2' is , R_3' is not COOH, COY"
or COO⁻,

wherein when R_2' is , , or ,
R_3' is not -NO₂, and wherein -R_2'-R_2'-R_3', collectively,
are not -H, sufficient to effect said protection.

38. A method of modulating NO· function as a
neurotransmitter in a mammal comprising contacting said
mammal with an amount of an oxidant scavenger of the
formula,

or a pharmaceutically acceptable salt thereof,
wherein:

R_1 is a bond, , , , ,
 , , , or wherein X is a
halogen and Y is an alkyl group and wherein
indicates bonding to R_2 at any position and
indicates bonding to R_2 and the substituent at any
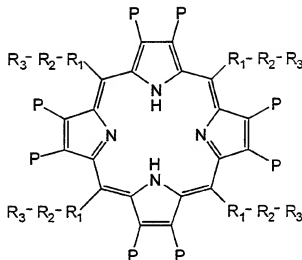position; and

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R_2 is a bond, $-(CY'_2)_n^-$, $-(CY'_2-CY'=CY')_n^-$, $-(CY'_2-CY'_2-CH=CH)_n^-$, $-(CY'=CY')_n^-$, or $-(CY'_2-CO)_n^-$, wherein Y' is hydrogen or an alkyl group and wherein n is 1 to 8; and

R_3 is $-Y''$, $-OH$, $-NH_2$, $-N^+(Y'')_3$, $-COOH$, $-COO^-$, $-SO_3H$, $-SO_3^-$, $-C-PO_3H_2$ or $-C-PO_3H^-$, wherein Y'' is an alkyl group,

optionally complexed with a metal selected from the group consisting of manganese, copper and iron, sufficient to effect said modulation.

39. A method of modulating NO· function as a neurotransmitter in a mammal comprising contacting said mammal with an amount of an oxidant scavenger of the formula,

or a pharmaceutically acceptable salt thereof, or metal complex thereof wherein said metal is selected from the group consisting of manganese, copper and iron, wherein:

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each R_1' is independently a bond, [phenyl with CO_2Y''], [phenyl with OH],

[phenyl], [phenyl with Y''], [phenyl with CO_2H], [phenyl with $\overset{O}{\overset{\|}{C}}$-NH-], [phenyl with $\overset{O\ O}{\overset{\|\ \|}{C}-C}$-], [phenyl with $\overset{O}{\overset{\|}{C}}$-] or

[phenyl with $\overset{C(Y'')_3}{\underset{C(Y'')_3}{}}$], wherein Y" is an alkyl group, and wherein [phenyl]

indicates bonding to R_2' at any position and [phenyl]

indicates bonding to R_2' and the R_1' phenyl substituent

at any position;

each R_2' is independently a bond, or $-(CH_2)_n-$
wherein n is 1-4,

each R_3' is independently -Y", -Y'", -H, -OH, -OY",
$-NO_2$, -CN, $-NH_2$, -COOH, -COY", $-COO^-$, or a heterocyclic
group, wherein Y" is as defined above and Y'" is a
primary, secondary, tertiary or quaternary amine,

wherein when R_1' is [phenyl with $\overset{O\ O}{\overset{\|\ \|}{C}-C}$-] , R_3' is not COOH, COY"
or COO^-,

wherein when R_1' is [phenyl with $\overset{O}{\overset{\|}{C}}$-NH-], [phenyl with $\overset{O\ O}{\overset{\|\ \|}{C}-C}$-], or [phenyl with $\overset{O}{\overset{\|}{C}}$-],
R_3' is not $-NO_2$, and wherein $-R_1'-R_2'-R_3'$, collectively,
are not -H, sufficient to effect said protection.

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40. An oxidant scavenger of the formula:
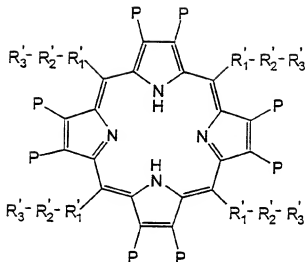

or a pharmaceutically acceptable salt thereof,

wherein:

R_1 is a bond, , , , ,

, , , or wherein X is a

halogen and Y is an alkyl group and wherein

indicates bonding to R_2 at any position and

indicates bonding to R_2 and the substituent at any

position; and

R_2 is a bond, $-(CY'_2)_n{}^-$, $-(CY'_2-CY'=CH)_n{}^-$, $-(CY'_2-$
$CY'_2-CH=CH)_n{}^-$, $-(CY'=CY')_n{}^-$, or $-(CY'_2-CO)_n{}^-$, wherein Y'
is hydrogen or an alkyl group and wherein n is 1 to 8;
and

R_3 is $-Y''$, $-OH$, $-NH_2$, $-N^+(Y'')_3$, $-COOH$, $-COO^-$,
$-SO_3H$, $-SO_3{}^-$, $-C-PO_3H_2$ or $-C-PO_3H^-$, wherein Y'' is an
alkyl group,

wherein, when R_1 is and R_2 is a bond, R_3 is

not Y'' (eg $-CH_3$), and

wherein, when R_1 is and R_2 is a bond, R_3 is

not $-Y''$ (eg $-CH_3$), $-N^+(Y'')_3$ (eg $-N^+(CH_3)_3$), or COOH.

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41. An oxidant scavenger of the formula:

or a pharmaceutically acceptable salt thereof,

wherein:

R_1 is a bond, -H-, -⟨⟩-, -⟨⟩-N±, -⟨⟩-NO_2,

-⟨⟩-SO_3H, -⟨⟩-SO_3^-, -⟨⟩-X, or -⟨⟩-Y wherein X is a

halogen and Y is an alkyl group and wherein -⟨⟩-

indicates bonding to R_2 at any position and -⟨⟩-

indicates bonding to R_2 and the substituent at any

position; and

each R_2 is independently, a bond, $-(CY'_2)_n^-$,

$-(CY'_2-CY'=CY')_n^-$, $-(CY'_2-CY'_2-CH=CH)_n^-$, $-(CY'=CY')_n^-$,

or $-(CY'_2-CO)_n^-$, wherein Y' is hydrogen or an alkyl

group and wherein n is 1 to 8; and

each R_3 -is, independently -Y", -OH, -NH_2, -$N^+(Y")_3$,

-COOH, -COO^-, -SO_3H, -SO_3^-, -C-PO_3H_2 or -C-PO_3H^-,

wherein Y" is an alkyl group,

each P is, independently, an electron withdrawing

group or hydrogen wherein at least one P is not

hydrogen, or

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or a pharmaceutically acceptable salt thereof,
wherein

each R_1' is independently a bond, , ,

, , , , or

, wherein Y" is an alkyl group, and wherein

indicates bonding to R_2' at any position and

indicates bonding to R_2' and the R_1' phenyl substituent

at any position;

each R_2' is independently a bond, or $-(CH_2)_n-$
wherein n is 1-4,

each R_3' is independently -Y", -Y'", -H, -OH, -OY",
-NO$_2$, -CN, -NH$_2$, -COOH, -COY", -COO$^-$, or a heterocyclic
group, wherein Y" is as defined above and Y'" is a
primary, secondary, tertiary or quaternary amine,

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each P is, independently, an electron withdrawing group or hydrogen wherein at least one P is not hydrogen;

wherein said oxidant scavenger is a metal complex wherein said metal is selected from the group consisting of manganese, copper or iron,

wherein when said metal is copper and all P's are Br, and when R_1 is ⟨◯⟩N- and R_2 is a bond, R_3 is not $-CH_3$.

42. The oxidant scavenger according to claim 41 wherein said scavenger is a manganese, copper or iron complex of

octobromo - TBAP
octochloro - TBAP
octofluro - TBAP
octonitro - TBAP

octobromo - TMPyP
octochloro - TMPyP
octofluro - TMPyP
octonitro - TMPyP

octobromo - tetrakis 4-phenyl sulfonate porphyrin
octochloro - tetrakis 4-phenyl sulfonate porphyrin
octofluro - tetrakis 4-phenyl sulfonate porphyrin
octonitro - tetrakis 4-phenyl sulfonate porphyrin.

43. A method of protecting a biological substance from oxidative stress or a method of maintaining biological function of NO·, or active forms thereof, in an intracellular or extracellular space of a tissue or organ comprising contacting said substance or said tissue or organ with an amount of said scavenger

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according to claim 41 sufficient to effect said
protection or said maintenance.
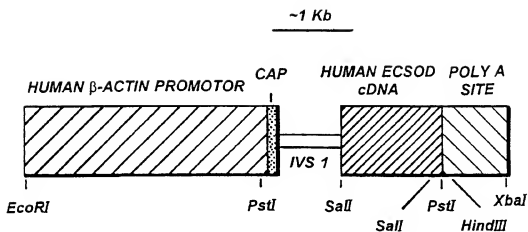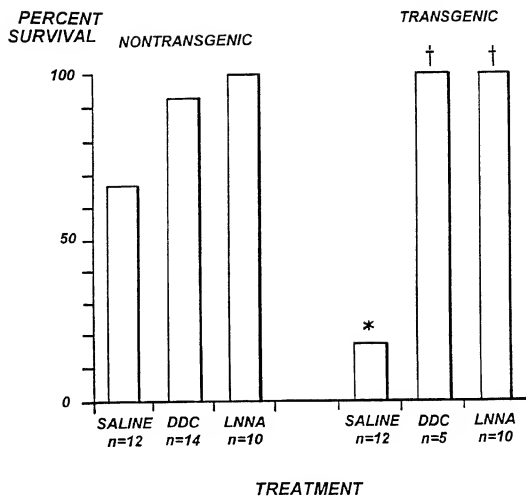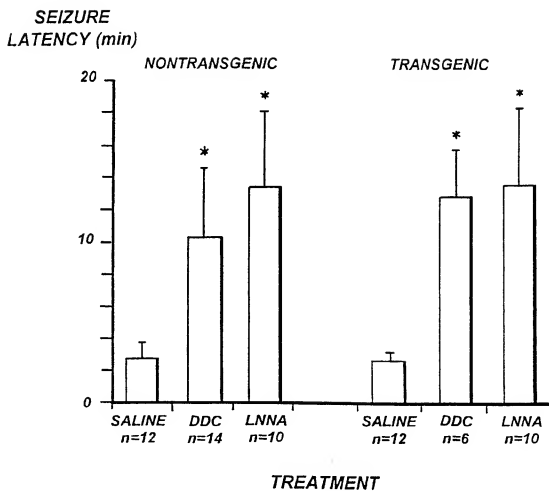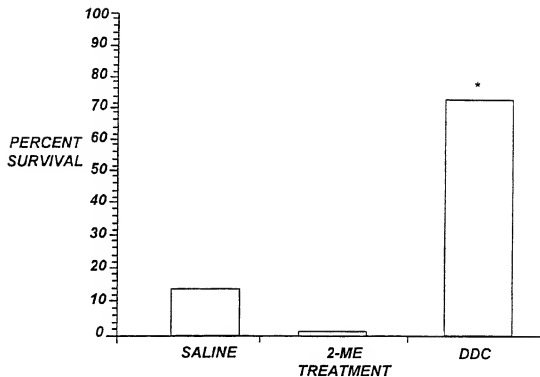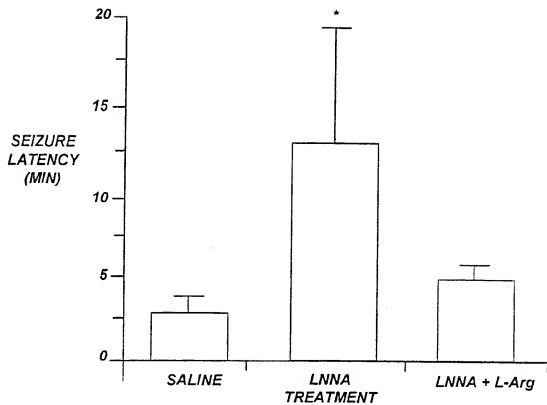
FIG. 1

FIG. 2

FIG. 3

FIG. 4

FIG. 5

FIG. 6
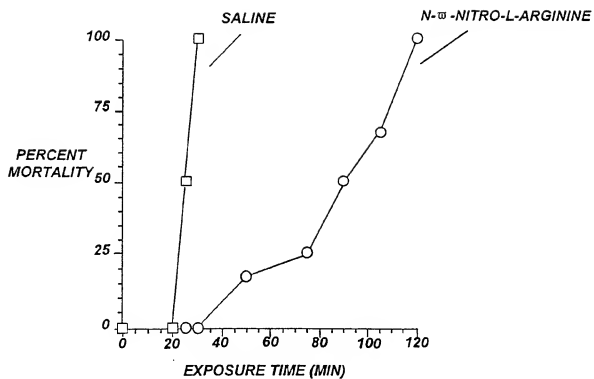
FIG. 7
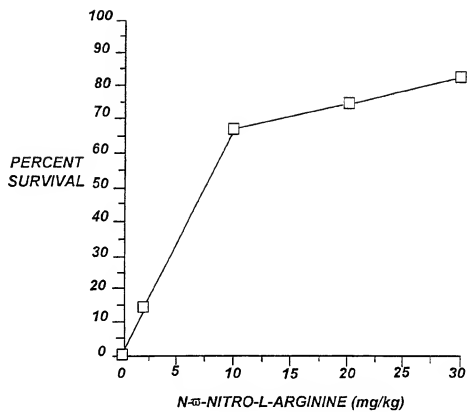
FIG. 8
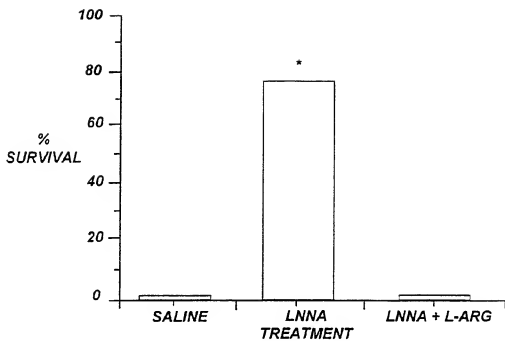
FIG. 9
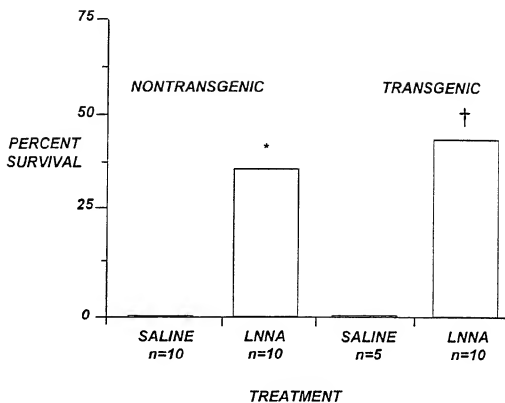
FIG. 10
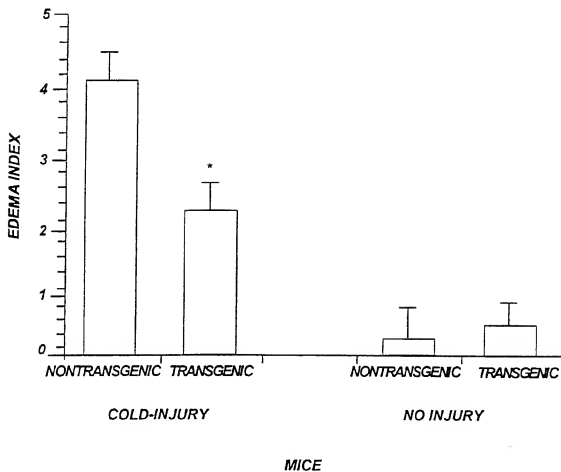
FIG. 11

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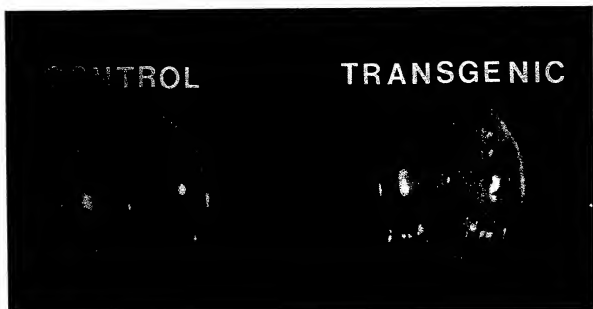
FIG. 12

FIG. 13
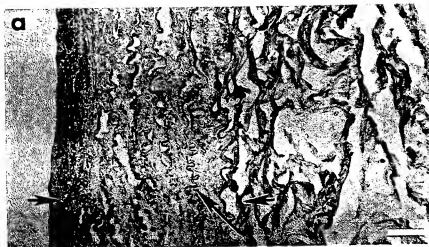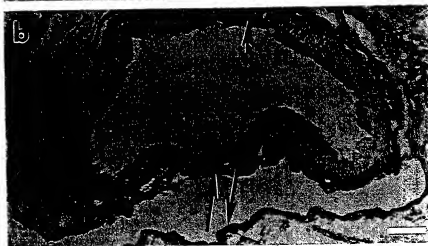
FIG. 14A

FIG. 14B

FIG. 14C
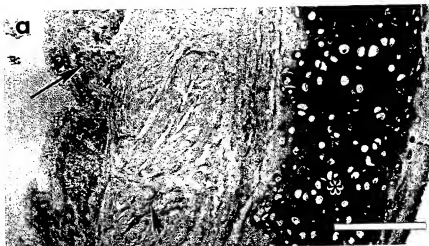
FIG. 15A

FIG. 15B

FIG. 15C
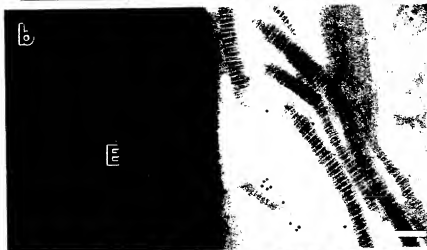
FIG. 16A

FIG. 16B

FIG. 16C

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FIG. 17

FIG. 18A

FIG. 18B

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FIG. 19

FIG. 20A
RESTRICTION MAP
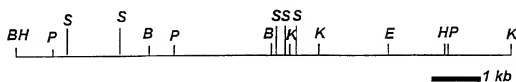
FIG. 20B
SEQUENCING STRATEGY
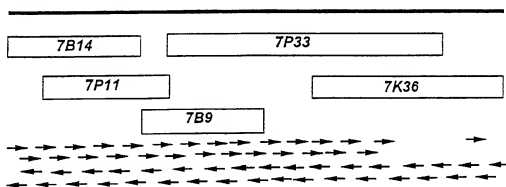
FIG. 20C GENE STRUCTURE
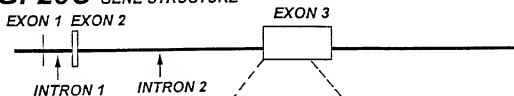
FIG. 20D
PROTEIN STRUCTURE
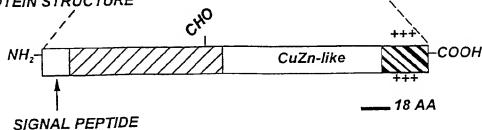
SUBSTITUTE SHEET (RULE 26)

FIG. 21A

HEART BRAIN PLACENTA LUNG LIVER SKELETAL MUSCLE KIDNEY PANCREAS

-7.6

-4.4

-2.4

-1.35

FIG. 21B

RELATIVE ABSORBANCE
(ARBITRARY UNITS)

19.1
13.6
7.5
4.1 4.7
1
2.2
6.3
10.2

HEART BRAIN PLACENTA LUNG LIVER SKELETAL MUSCLE(1.4kb) SKELETAL MUSCLE(4.2kb) KIDNEY PANCREAS
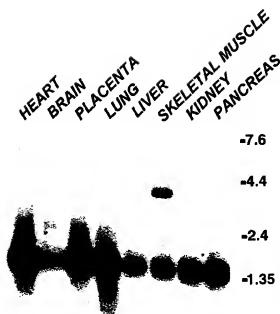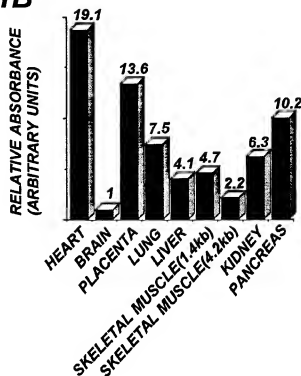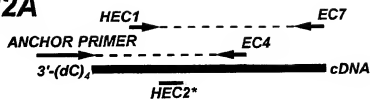
FIG. 22A

FIG. 22B
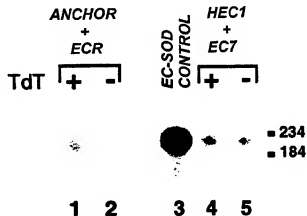
FIG. 23
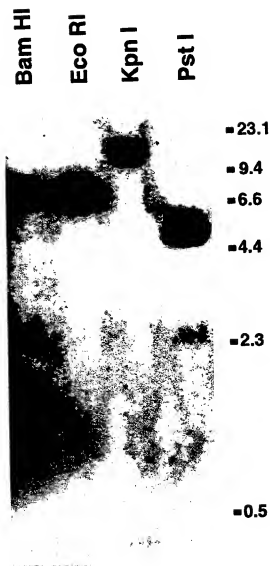
FIG. 24

```
GGATCCAGAG ATTTAGATTT TTTATAAGCT TTCCTGCCAC CGAAACGGGT GTTTGGGACC     60
TCACGAGGCC CTGTTCATTC TTCGTCGCTG CGCTCCCCAC TCTGTACTGG ATGCATTTAC    120
TGACGTTGTT GTCTCCGTCC CCAGAGTATG AACCCCCAAG GTGACTCATG CAGCTGTGGG    180
TGCCCGGCAT ACAGCATGGT GACTGGAATG GATGAGCACC CAATAAACAT TTGTTGCAGG    240
AATGCAGGAG GACGGGCAGG CCAGCAAGCA GGCTGCCTGG TTTTTCCCAC ATGGGCTTTT    300
CTGGGAAAGA AGAGCTTCTA TTTTTGGAAA GGGCTGCTAT GATTGAGAAA AGTTCATGGC    360
AGCAAAAAAA GGACAGACGT CGGGAGGGAA ACACTCCTAG TTCTCCCAGA CAACACATTT    420
TTTAAAAAGA CTCCTTCATC TCTTTAATAA TAACGGTAAC GACAATGACA ATGATGATTA    480
CTTATGAGTG CGGCTAGTGC CAGCCACTGT GTTGTCACTG GGCGAGTAAT GATCTCATTG    540
GATCTTCACG GTGGGCGTGC GGGGTGGACA GCCTCACACC CCCATTTTAC AGATGATGAA    600
AAGGAGGTGC AGGGAGTGGT GCAGCTGCTT CAGGCGTACA CAGATAGGAA GTGACAAGGC    660
TGGGACTCTG CAGCCTGAGT GTGTCATCAC GACCCACCCG CTGCTCTGCT CTCATAGGTA    720
TGACAGCACA GCTCTGGAGC AAATGCCATG CACATTTGCA AGGTGCCCAT TTCCATGCAG    780
CAAAAATAAG TCAATAAGTT ATTGACTTAG AGAAAAGCAA AGGGCCTCTC AATAAAGAGG    840
TCATTGTACA CCTCTCCAAA CAGGCGATTT TCTTTCTCAT TTTTATTCCC CTGCTGTGTG    900
CTGAAGGTCA CTGGCTACAA GCCGGTGAAG TCGCGGAATG GAATCCTTGG CCCGAAAACC    960
CAAAAATGGG AGGGGCAGAG GAGGTGGGGA CAGAGCGGGA GGAGGTGGAG GCGAAGCAAT   1020
TCTACAACCC GGGGAGGTCT GGCCTGCTTT TCCTCCCTGA ACTGGCCCAA TGACTGGCTC   1080
CCTCACGCTG ACCACTCCTC TGGGCTGGCC TCCTGCACTC GCGCTAACAG CCCAGGCTCC   1140
AGGGACAGCC TGCGTTCCTG GGCTGGCTGG GTGCAGCTCT CTTTTCAGGA GAGAAAGCTC   1200
TCTTGGAGGA GCTGGAAAGG TGGGTGCTAA GTTGAGGTTC ATTTTGTTCT TCTCGGAGTG   1260
TGCTTATTGA GTCTGAAGCT GGGGTTGGGGC AACGGGCCTC TTCTTGGGAA CAAATTGGAT   1320
CATCTTCTTG GGAAGGAAAT GTACTTTCCC TGGCTGCTCT GAGGGGTTAG TGGGGAGGTG   1380
GAGTGAGCGG GGAGGAAGGC AAGGAGGGGA GGAAGAAACC GTTCCTCCTG TGGATCTGCA   1440
AAGACCAGTC CAAGAGGATT TTAGTGTTAG GAAAAGGAAT CTGGAGTGAC GAGAAAGGGG   1500
GCCTTTCTAG ATGTTGCATG GCTTTGGTGT CGGGAGCCAC TTATGGGACA GCAGGTACTC   1560
TAAAAAGCCA CCTCCTTAGG AAAGCAGAGA GGCCCTGGCC AGCTCAGGCT CCCAGCAAGA   1620
GCTCCTTCTA GGAGACAGCT GAGGGATGAA ACACACCCAA GGCTCAAGAG GGGCAGGTTC   1680
TTCCCAGATA CAGACCCAGG AAGGAGATAA AGGCTTGGTG CCTCTATTTG GTTCAGGATA   1740
AGGGCCCCTG TCCTCTTTCT CTGATAACAC TGTCCTCTTT CTCTGATAAC ACCGTCCTCC   1800
CTTCCAGATC CACGTACAAA GGAGGCCCTT AAAAAGGCAC TTGGTCATTC ACAGCTCAAA   1860
CTGAGCAAGA GGCTGTGGGA GAAGAATCAA GTTGGTCCCG AGGGGAAGAG GTGTCAAAGG   1920
CTTAAGAAAC AAGAAGTCAG AGTTTACCTG GGTTTGAGGG AGAATTTTCT TTCCCCCTTT   1980
```

FIG. 24(cont.)

```
TCCTCCTCCT CCTCCTTCTT CTCTTTTTTT TTTTTTTTTT TTTTTTTTTT TTGAGACATG  2040

GTCTCATTCT GTCACCCAGC ACCCAGGCTG GAATGTAGTG GCACGATCAC TATCACGGCT  2100

CACTACAGCC TCTACCTCCC GGGCTCAAGT GATCCTCCTA CCTCAGCCTC CTGAGTAACT  2160

GGGACTACAG GCACATGCCA CCACACCCAG CTATTTTTTT TTTTGCTAGA GATGGGGGTC  2220

TCTACCAGGT TGGTCTCATA CTCTTGTACT CAAATGATTC TCCTGTCTCA TCCTCCCAAA  2280

GGGTGGGATT ACAGGCATAA GCCACCATGC CTGGCTCTTC TTTTGGTTTC AGAGAAAAAC  2340

ATCTCCTTAA AATGTTTATT TCCCAAGGAT TCTTGAAAAA GAAAGCTCAC TGACACACCC  2400

AAAACAATCT GGTTTTGCTC TGTGCTTTTA GGGAGAACTT TCTAAGCAGC AGAGCCCTTC  2460

TGAGTGGCAG GGCTGTCTTA GGAGGAAGGT GTCTTTTGAT GATGGGGAAC TTCATGTCCA  2520

GGTCTGGCAG GAGAGTTACC CCACTTTCCT GCCTACTCCC TGGGGCTTTG GGGTAGTAGT  2580

ACCACATTGG GCCATGTCAT TTAGGTGAGT CCTTCAACAT CACTTTCTCT GCTTCTCCCT  2640

CTTTCTGGAT CCTCCTTCTT GGAGCCTTTC AAGGGGACCT CCTCTCACAG TGTCCATAGC  2700

ATCTCTTAGC TAATGGTCCT TAAAATCTCT ACCAGCAGCT TCTCTCTGAT AGCTAAGAGC  2760

TGCCATTTAC TGGGAACTTT CTATGTACTG GGCTCTGTGC TAAGTGCCCT AGATGAGAGA  2820

TGTGCAGTGT GGTGCCTAAA CCTTGGGCTT GGAGCAGACA CACACTTTCA AATCCTGCCT  2880

TCAGCTCCTT AGTGAACATG TCACCTTGGG CGGGACACAC GCCTCTCTGT GCCTCAGTTT  2940

CCTACACTTT AGAATGGGGA TAACACTGAA TAATGTTCTT GTGAGGATGC AGGGAATTAA  3000

CCCACGCACA GTACTTATAA TAGTGTCTGG CGCCTGTGTT CGATAAGTTT TAGCAATTCT  3060

AATCATCTCT TTTAAGCCTC GCAGCAAGCC TCTAAGGTAA GTCTGTATTA GTATCCCTAT  3120

TTACAGATGA GAAAACTGAG GTTCACAGGG GATGAGACAG TGTACAGTCT GCAGTCCAGC  3180

AATTACTCTG CTACTCAGCA ATAAAAATAG TAACAGCTAA CCCTTAGACT AAGTGGCAGA  3240

GTCAGGCTTT AGATTCATGA GGTGAGTTCT GGAATCCATC CCTTTAATAA CCACACTAAA  3300

TTGCCTTTCT GAAATGGTTA TATAAAGCAT ATCTACCCAA TCTTGGAGTT TTTTAAATGG  3360

CACCTAGTTT GGTGCTGGAA ATGCAGTTGA CCTTCAAAGC AATTCTTTGG AGGCAGCATC  3420

AATCCCTCTG GAAATACCTC GGTGGCATGG CTGGCCTTAT TCTACAGGTA AGGAACTTGA  3480

AGCTAAGCAT CAGTAACCCC GTGAAGTCAC AGTTAGTATA GGTTGGAATT GGGATTCAAA  3540

TCTGTACCTG ACTTTATAAT TCCTAGCTGG GCCCCAGAAT CTTTGATAGA GGTGTCTTCT  3600

TTCTTTTCTT TTCTTTCTTT CCTCTTTCTT TCCCTTCCTT CCTCTCTCTC TGTCTTTCTT  3660

CTCTCCTTTC TTTCTCACAG AATCAAAATC TCTTGGGGTG GGGCTGGGC ATCTGATTTT  3720

TAAAAACCAG ACATCTGATG TGCAGTCAAC ACTGAGAACC CCTGCCAGCT TCATCTCCTC  3780

TTCTAAGTGC CAGACCCAAG TTTCCAACTG TCTGCCCACC TGTCTCCCCA CCTGGGCACC  3840

CGCCAGCGTC TCACCCTCAG GAGACTCCAG CTGAACTAAT CCTCTCTCCC TGCTTTTCCA  3900

GAACAGGTCC CACCCTCCCT CCACTCAGTC TCTCCTGCTG GGAACCCTGG TCATCTGCAC  3960
```

FIG. 24(cont.)

```
TGTGCCTTCA TCTTCCATCC TGCCAGTGCT GCCCGGTGTG TCTCTTAAAC CCATGCCTCC        4020

TCTGTGTGCA CCACCTGCAC TTTGGTAAAA GCCTTCATTT CCTGCTTGGG TTACTACAAC        4080

GCCCCCTAAC TCATCTCACT GTCTCTATTT CTGCTTCTCT GTCTCTCCCT AGGCTACTCC        4140

CATTCTTCCT CCCCTTTCCT CTTCATCCCA AAGTCCAACC CATATCCTTT TACCAGTAGG        4200

ACTTAAGGAA CTAAAGACTA TCTCATCACC CACTTTTCTT CTTAAAAACT TCCACTGCAC        4260

TGCCTGCTGA GATGGCCTTC CTACCCAACT TGGCTGGAAA ACTCCTACCC ATCTTGTGGA        4320

ACCCAGTTCA AAAGTCACCA CCTCTGAGAA GCCTTCCCTG AGGCTCCTAG GGAGATGGGT        4380

ACTGCCTCCT CTGTCCTTCT CCAGCACAGG CCCCATCTTC AATCACAGGA TTGTGCTGGA        4440

ATGATTGGAT GCCAAGTCTG TCCCTCACTG AACTCCTTAT GCAAAATCCA TATTATATGT        4500

TTCCTTTTGC CAGGTGTGGG CCCAGGTGCT GGGGATACCG ATGAATAAAA CTGAGTTTCT        4560

GTCTTCAAGA AGCTCCAAGT CTACTGAGTG TAGCAGAGAA CAGGGAGAGG GCACTTCAGG        4620

GAGAAGGGGT AGCACATGCA AAGCCCCAGA AGGCAGGGAC AGAAGCCTTA GGGATGTCTG        4680

TGGGGGAGGA TGGAGGAAGA GGGTAACAGG AGACCAGGTG GGGAGATGAG GGAGGTGGTC        4740

TGGAAGGGCC ATGAGACACC CCTCACGCTC CCTGAGACCC CCTCCACGCT ATAGAGATGG        4800

GACTGGAGAG GACGATGATC ATTTGTGACT CAGATCCCTG TGGGTTTCTT CAGATTGGGT        4860

CTCACCCATC TTTACAGCCA CAGCACCTAA CACAGTGCCC GGCACACAGC AGGCCCTAGA        4920

CAAACGTTTG CCACATGAAG TCATGCCACT GGCCAGGAAG CCCACTGGGG ACTGGGGGGT        4980

TGGTTCTGCG ATAATGGGGT CCCTGAGATT CTATGTTTCA CGTGACTAAG CCTCACTCTG        5040

CCCCCACCTC CGCGGGGGCG TCCCGCAGGT GCCCGACTCC AGCC ATG CTG GCG CTA        5096
                                                Met Leu Ala Leu
                                                 1
```

```
CTG TGT TCC TGC CTG CTC CTG GCA GCC GGT GCC TCG GAC GCC TGG ACG        5144
Leu Cys Ser Cys Leu Leu Leu Ala Ala Gly Ala Ser Asp Ala Trp Thr
 5                  10                  15                  20
```

```
GGC GAG GAC TCG GCG GAG CCC AAC TCT GAC TCG GCG GAG TGG ATC CGA        5192
Gly Glu Asp Ser Ala Glu Pro Asn Ser Asp Ser Ala Glu Trp Ile Arg
                25                  30                  35
```

```
GAC ATG TAC GCC AAG GTC ACG GAG ATC TGG CAG GAG GTC ATG CAG CGG        5240
Asp Met Tyr Ala Lys Val Thr Glu Ile Trp Gln Glu Val Met Gln Arg
            40                  45                  50
```

```
CGG GAC GAC GAC GGC ACG CTC CAC GCC GCC TGC CAG GTG CAG CCG TCG        5288
Arg Asp Asp Asp Gly Thr Leu His Ala Ala Cys Gln Val Gln Pro Ser
            55                  60                  65
```

```
GCC ACG CTG GAC GCC GCG CAG CCC CGG GTG ACC GGC GTC GTC CTC TTC        5336
Ala Thr Leu Asp Ala Ala Gln Pro Arg Val Thr Gly Val Val Leu Phe
        70                  75                  80
```

```
CGG CAG CTT CCG GCC CCC CGC GCC AAG CTC GAC GCC TTC TTC GCC GAG        5384
Arg Gln Leu Ala Pro Arg Ala Lys Leu Asp Ala Phe Phe Ala Leu Glu
85                  90                  95                  100
```

```
GGC TTC CCG ACC GAG CCG AAC AGC TCC AGC CGC GCC ATC CAC GTG CAC        5432
```

FIG. 24(cont.)

```
Gly Phe Pro Thr Glu Pro Asn Ser Ser Ser Arg Ala Ile His Val His
                105                 110                 115

CAG TTC GGG GAC CTG AGC CAG GGC TGC GAG TCC ACC GGG CCC CAC TAC     5480
Gln Phe Gly Asp Leu Ser Gln Gly Cys Glu Ser Thr Gly Pro His Tyr
            120                 125                 130

AAC CCG CTG GCC GTG CCG CAC CCG CAG CAC CCG GGC GAC TTC GGC AAC     5528
Asn Pro Leu Ala Val Pro His Pro Gln His Pro Gly Asp Phe Gly Asn
        135                 140                 145

TTC GCG GTC CGC GAC GGC AGC CTC TGG AGG TAC CGC GCC GGC CTG GCC     5576
Phe Ala Val Arg Asp Gly Ser Leu Trp Arg Tyr Arg Ala Gly Leu Ala
    150                 155                 160

GCC TCG CTC GCG GGC CCG CAC TCC ATC GTG GGC CGG GCC GTG GTC GTC     5624
Ala Ser Leu Ala Gly Pro His Ser Ile Val Gly Arg Ala Val Val Val
165                 170                 175                 180

CAC GCT GGC GAG GAC GAC CTG GGC CGC GGC GGC AAC CAG GCC AGC GTG     5672
His Ala Gly Glu Asp Asp Leu Gly Arg Gly Gly Asn Gln Ala Ser Val
                185                 190                 195

GAG AAC GGG AAC GCG GGC CGG CGG CTG GCC TGC TGC GTG GTG GGC GTG     5720
Glu Asn Gly Asn Ala Gly Arg Arg Leu Ala Cys Cys Val Val Gly Val
            200                 205                 210

TGC GGG CCC GGG CTC TGG GAG CGC CAG GCG CGG GAG CAC TCA GAG CGC     5768
Cys Gly Pro Gly Leu Trp Glu Arg Gln Ala Arg Glu His Ser Glu Arg
    215                 220                 225

AAG AAG CGG CGG CGC GAG AGC GAG TGC AAG GCC GCC T GAGCGCGGCC        5815
Lys Lys Arg Arg Arg Glu Ser Glu Cys Lys Ala Ala
    230                 235                 240

CCCACCCGGC GGCGGCCAGG GACCCCCGAG GCCCCCCTCT GCCTTTGAGC TTCTCCTCTG   5875

CTCCAACAGA CACCTTCCAC TCTGAGGTCT CACCTTCGCC TCTGCTGAAG TCTCCCCGCA   5935

GCCCTCTCCA CCCAGAGGTC TCCCTATACC GAGACCCACC ATCCTTCCAT CCTGAGGACC   5995

GCCCCAACCC TCGGAGCCCC CCACTCAGTA GGTCTGAAGG CCTCCATTTG TACCGAAACA   6055

CCCCGCTCAC GCTGACAGCC TCCTAGGCTC CCTGAGGTAC CTTTCCACCC AGACCCTCCT   6115

TCCCCACCCC ATAAGCCCTG AGACTCCCGC CTTTGACCTG ACGATCTTCC CCCTTCCCGC   6175

CTTCAGGTTC CTCCTAGGCG CTCAGAGGCC GCTCTGGGGG GTTGCCTCGA GTCCCCCCAC   6235

CCCTCCCCAC CCACCACCGC TCCCGCGGCA AGCCAGCCCG TGCAACGGAA GCCAGGCCAA   6295

CTGCCCCGCG TCTTCAGCTG TTTCGCATCC ACCGCCACCC CACTGAGAGC TGCTCCTTTG   6355

GGGGAATGTT TGGCAACCTT TGTGTTACAG ATTAAAAATT CAGCAATTCA GTACTGCGTC   6415

GAGGTCTTGG TTACTTTTTT GTTTGTTTGT TTTAGGCTTC TCTCCCAAGC TGAGCTTTTT   6475

TTTGTTTTGT TTTCGTTTTC CTTTTTTTTC TTTTTTTTGG GAGTGGCAAA CATGCTTCCC   6535

AAATCCCTAC AGGACTTCTC CTTATCCTCT GCCCCCACCT CCCTAACCCT GCTGGCAACA   6595

ACGTTCAGCC ACTGCTTGTC TTGCCCTTCA GTGTGGCTCC AAGAGGAAGA TCACCAGAAT   6655

CACTCAGGGA AGTTAAAAAA AAAAATACAG CTTCCTGGGC TACATCCCAG AGCTGTGGAA   6715
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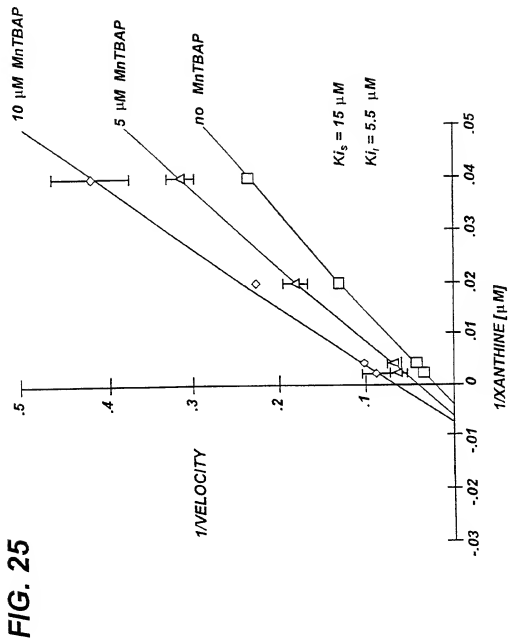
FIG. 24(cont.)

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TCCAAAGGGA GAAGAGAAAG TGAATTTGCG ACAAGCGTCG GGATGATTCT GGCACTGGAC   6775

CCTCTGGCCT GAGAGGGGAA GAGGCCTTCC ATCTCACCTG GGCTGGTAGC TTGTCACATC   6835

TGCCTCCGAG TACAGCCTTA GGTCCATTTC CCAGATATCA GAGACAGTGC CAGGGAAGCC   6895

AGGTGACTGC ATCTTGCCTA GGCACAGAAG AGTAGGGTTG GAATGTGACG TTGTTAGCAT   6955

TTGGCAGGAC CAAAACCAGA GGCAAACGGA GGCAGTGGGA TGGAAAGGCA GTTGATTTTG   7015

ATGAAGGCTT GTTGGGAGTT CAGCTTTCTT TTGAAACTTA TAATCTATAC CCAGGCTAGA   7075

ACAGTCTTGT GTATACACCT TCATTCATGG AATAAACGTA CTTGCAATAA CTTTTTAGCC   7135

TCCCAGGGTA GCCTCACTTC CTAGCTGTGA CTTTTCCACC CTGGTTACTG GGAGGCAGCT   7195

TCCATTTCTC CCAGACTAGC TAGGCAGTGC GTCCAACTGA ACCGCAGCCA GAAACCTGTC   7255

TCCAGGGGTT ATTTTTACCT CTAACTAGGA CTAACTTATT TTAAAATCTT TCCTTGAGCC   7315

CAAGTGACAA CTGAAGAGAA AGGCTATTGC CTGGTGATTT TGCTCCACCA GTTGGTTCTC   7375

ACTGGTTTGA ATACTAACTT GAACTGTACT CATCGACACT GAAAGGGGAT GAGCAAACAG   7435

TGTCTCTAAA TCTCCTGATC CTGATCTCAA ATATCCCCCT AATTACAAGT TGCAACAAGG   7495

CAGCTATTAC ACGGGGACAC AGGATGGAGA GGATGGGTGC CAAACACCCA TCGTCTACTC   7555

TGCTGCCTCG GTTATGGTGA ATTCAGGACC ATCAAGGGAG GTGTGGACCT TTTTTTTCAG   7615

AAGGAGGCTG ACACTTCTTG TCAATTGCAT TGTGTTCTTA GTTTTGCTCT TCACAACCCT   7675

TGACCCCGTA GATGGGGGCT GAAGAGGCAC CCTGGCCGAC TCACTCTATT TCTGTTTTGG   7735

GAATGGGATG GATAAACTAT CCCATGGCCT CCAGAGCCAA AAAACCAAAA CGAAACAAAA   7795

CAAAAAACCC CAAAACAAAA AAGCAAAAAG CAAACAAGAA AAAAAAAAAA AGAGGAAATA   7855

ATAGGCAGAC AATTTACAGT TCATTGTAAG GGCAAAGATA TGCATATAGC ATGATGGTTA   7915

ACAGGTCAGG CTCAGGTAGA AAGGCCCATT TGAACCCCAG CTCTGCCACA CTCAGAAACT   7975

GTGTGACCCG AACAAGTCAC TTAACCTCTC TGAGCATAGG TAAAATAAGA TCATCATACC   8035

AGATTGTTTT GAAGATTAAA TCAAGTGTTA TTCACGAGAG GTGCACAGCA TAGCATGCAC   8095

AACAAATAAG GACCTGGTAA GTATCTAATT AATAACAATG GCTAAGATCC AAAAAACAGC   8155

TACCTACTAA TAAATAGATG GGGCTGCCTT GTAAGGCAGT GAGCATCATG CAACCAGGAT   8215

TCAAATGAAG GACAGTTGCT ACCTCTGAGG TTCCCGAGAA GGATTTCTCG ATCCATTGAG   8275

AGACTGAATG ACATGAACTC TGCGATCCCA TCTCTTGTGG GGAGGGAACC TAGAATGAAG   8335

GGAAGATTGT GGGCCATAAA GGCAGACATC TGGTTCCTGG GCACAGAACC ATATGTGTGC   8395

CACCAAAGCC ACCCACCGGA CCCCACTTGG CCCCTGGAGT CTATTTTTAC TCCTCTCATC   8455

TTACAAGATC TATTTTGTTA ATCTCCTTAT ATTTGCTGTT TTGACTTCCC AGCCAGCTTG   8515

CTAATCAGTT TGCCTATTTG ACTCACAGGG TTTGCATTTG TCACGGGGAC TGAAACACAC   8575

GCTTGTTTTG ATTTCTTTTT GTAAATTAGA AGCGTTGATG TAATGACTCT ACCTAGACAC   8635

AGCTGGTAAA GTGAGAATAA TGCTCAAGTT TGCACAGTTT AAACACAATG TAGACAATAA   8695
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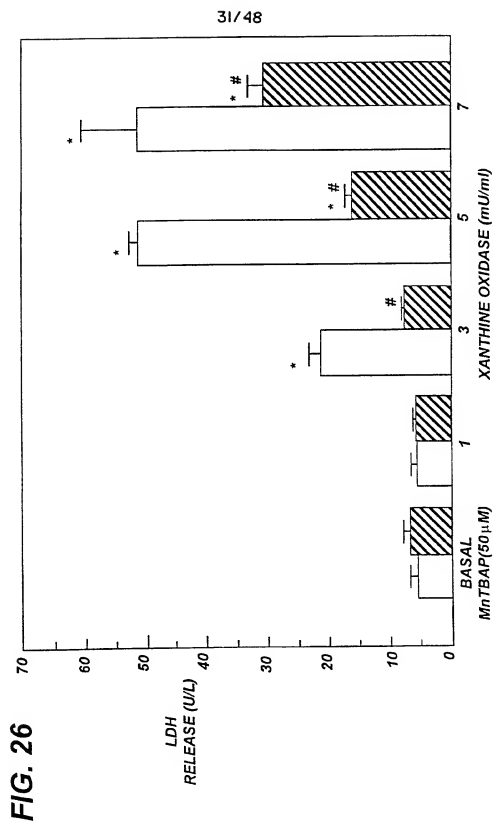
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FIG. 24(cont.)

```
TTAGAAATGC TATCTTTAGA TGTTTAGGAT AAGCTTTTCT CAGAATTGCA CTGATTTTTT    8755

TTTTCTGAGT GGGGCTTTTT AGTGCATATA TACAGAAATA CTAAAAACGT AAGAAAATAG    8815

AGCAAATCAG TGAGTGCTTT GGTCAACTTG AAAGACTGCA GGAAATAAAC CAACTGATTT    8875

TAGATCTGCC TTTTTTTGAC TGAATGCATA AAATCTTTAC ATTCTCCATA TTTTTCATGA    8935

CTACCATATG ATCAAATAGT TTTAGGTGAC AGATTGCAAC TGATAAGTTG CTGCAATATG    8995

GCAGAAGTCA TGCTCAGCCT CCGCTTGCCC GGTGGTGAGG GTGGAATATG AAGCAAACAA    9055

TAAAGATAAT TCATCATCTC TATCAGGAAA ATTGCCACAT GTTTATTTCA GGTAACAAAA    9115

AAGATATAGT TATGATATAC AATGAAACTA GAATCCAATA AAGCAACTTC TGCAAATGAA    9175

TAGAAGGTAC TTTTTCTTTA AATGAAACTA CAAAATAGCA GCTGGTTTTA AAAACAAAGC    9235

CAATTGTTTT AGATTTAATA GGCTACCACT GGCCTCTGCT AAGATCCCCA AATATATTCC    9295

TGAGCTCACA TAGATTCCAG AAAGTCAAAC TTTTCAATAT TATGCAAACT TTCCCTATGC    9355

ATCCAAAAAA TTCTCATTTA GTAAAGAGGT GATATGAAAT GTAAGGCAGC ATGTCCATAT    9415

CTATCATTTT AAATTGCCTT CATGCTGTAT CAACTGGTTT TGTTTTGGGA AGCAACCATA    9475

ATATTGAGAG ACGGGTCTTT CCTATTTTTT CTGCTACTCA TTTCTAACTA GATTCACTAC    9535

GGAGCTCCCA ATTGCATCTC TCTGATCTAC AAATTTTTCT CTCTTCAGGA AGACACCTGG    9595

AAAGAAGGGA CTACATTAAA GGAGTGTGTT GGGGGCAATG CTTTGGCCTT TTGACATCCT    9655

ATCTAGTCTG AAGGGACCCT CACTATTGCT AAGGAGGAGG AGTGTTTTAA ATGGAGGCTT    9715

CAGAATGAAA GCAGAGGAAG AAGGTACTCT CTTTTTCAAA AAGAAGGAGG GTACAGGCCG    9775

GGCGCAGCTG TCACGCCTGC AATCCCAGCA CTTTGGGAGG CCGAGGAAGG CAGATCACGA    9835

GGTTGGGAGT TTGAGCCAGC CTGGTCAACA TAGTGAAACC CCGTCTCTAC TAAAAATACA    9895

AAAATTAGCC AGCATGGTGG TGCATGCCTG TAGTCCCAGT TACTCGGGAG GCTGAGGCAG    9955

GAGAATCGCT TGAACTCGGG AAGTGGAGGT TGCAGTGAGC CGAGATCATG CCACTGCACT   10015

CCACCCTGGG TGACAGAGTG AGACTCTCAA AAAAAAAAAA AAAAAAAAAA AGAAGTAGGG   10075

TACC                                                               10079
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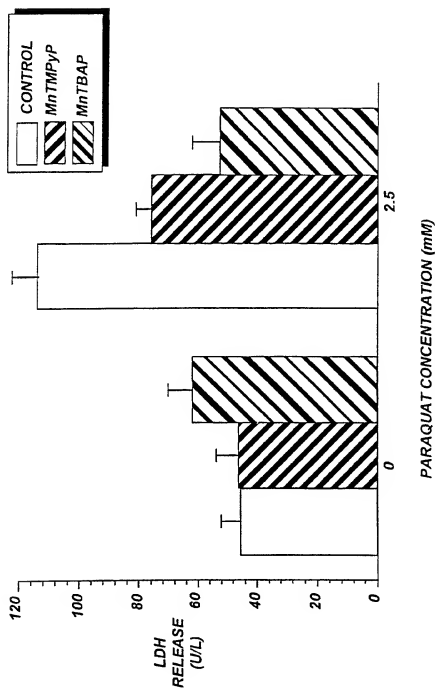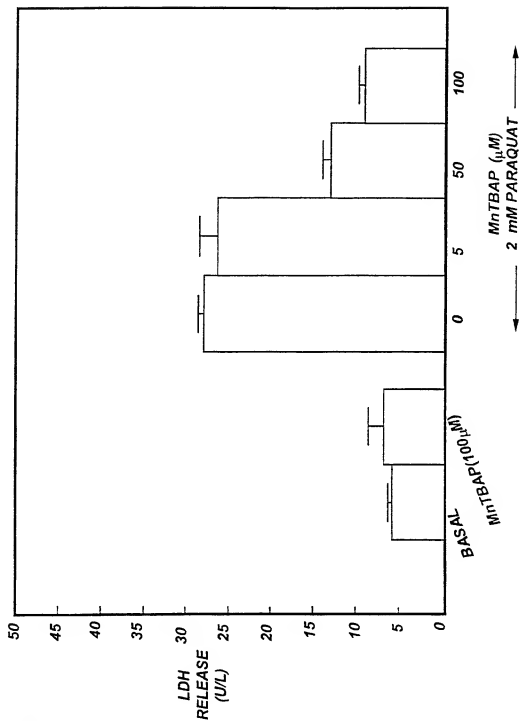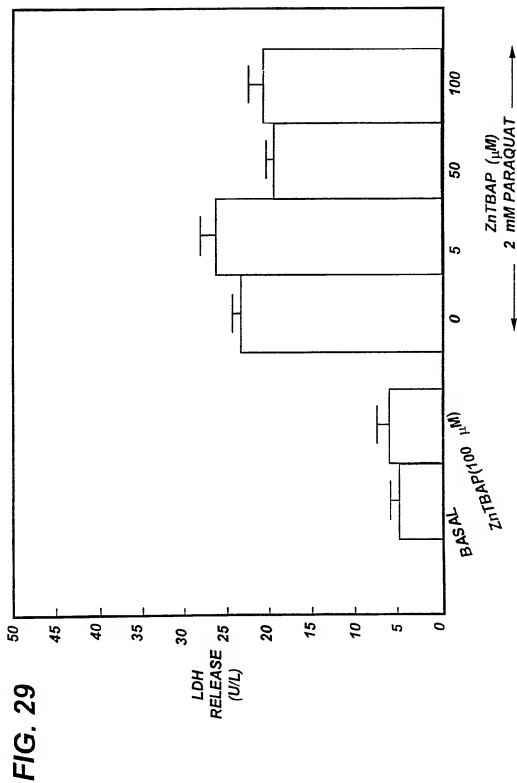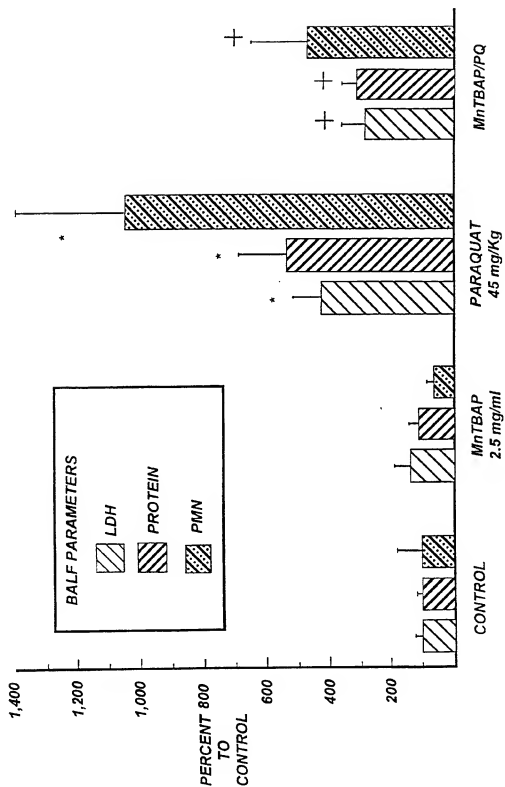
FIG. 25

10 µM MnTBAP

5 µM MnTBAP

no MnTBAP

$Ki_s = 15 \ \mu M$
$Ki_i = 5.5 \ \mu M$

1/VELOCITY

1/XANTHINE [µM]

FIG. 26

FIG. 27

FIG. 28

FIG. 29

FIG. 30

FIG. 31

FIG. 32

Log Glucose Oxidase (mU/ml)

Specific Cr^{51} Release

■ control
○ MnTBAP(100µM)

Glucose Oxidase LC$_{50}$ (mU/ml)	
CTR	MnTBAP
8	118

FIG. 37

LDH (% CONTROL)

Log [NMDA] µM

■ - MnTBAP
□ + MnTBAP

FIG. 33

(A) Percent Cr^{51} Release — MnTBAP / ←Glucose Oxidase (100mU/ml)→ — basal, MnTBAP, 0, 5, 50, 100 MnTBAP (µM)

(B) Percent Cr^{51} Release — MnTMPyP / ←Glucose Oxidase (100mU/ml)→ — basal, MnTMPyP, 0, 5, 50*, 100* MnTMPyP (µM)

(C) Percent Cr^{51} Release — ZnTBAP / ←Glucose Oxidase (100mU/ml)→ — basal, ZnTBAP, 0, 5, 50, 100 ZnTBAP (µM)

(D) Percent Cr^{51} Release — CuZnSOD / ←Glucose Oxidase (100mU/ml)→ — basal, CuZnSOD, 0, 50, 100, 500 CuZnSOD (U/ml)

FIG. 34A

FIG. 34B

FIG. 35A

FIG. 35B

FIG. 35C

FIG. 36A

FIG. 36B

FIG. 38A

PARAQUAT

FIG. 38B

NMDA

FIG. 38C

KAINATE

FIG. 39

p<0.025 Control vs. SOD Knockout

FIG. 40

FIG. 41A

FIG. 41B

FIG.41C

FIG. 42A

FIG. 42B

FIG. 42C

FIG. 42D

SOCl₂
beware
reflux

(15)

HN CH₃
CH₃

in 2M THF
solution
reflux for 6 hours

(16)
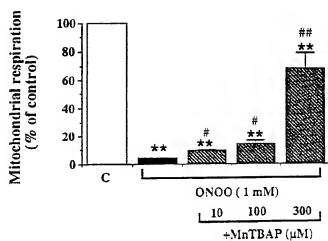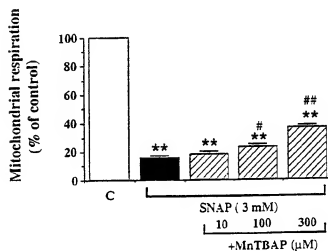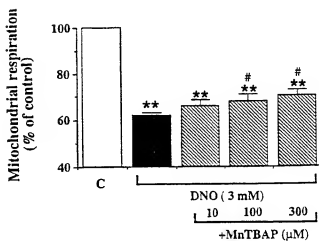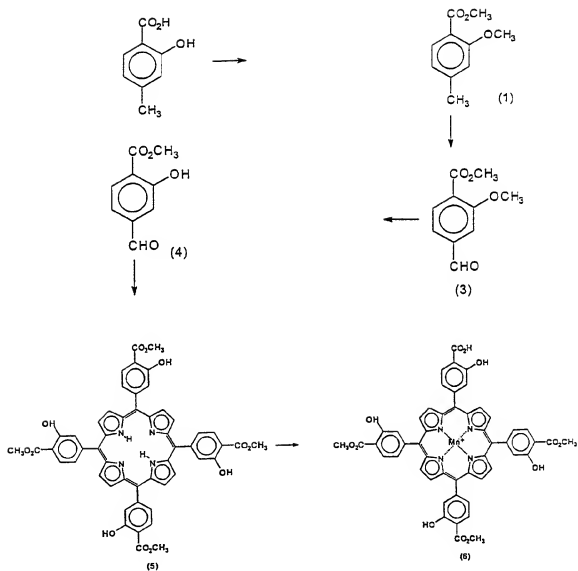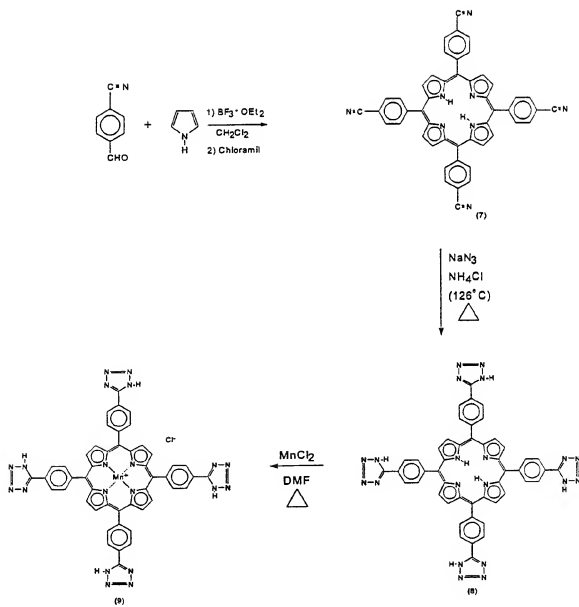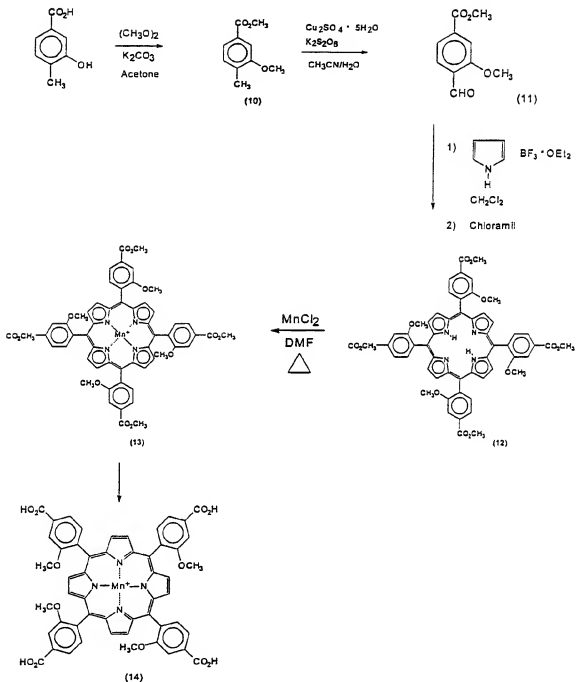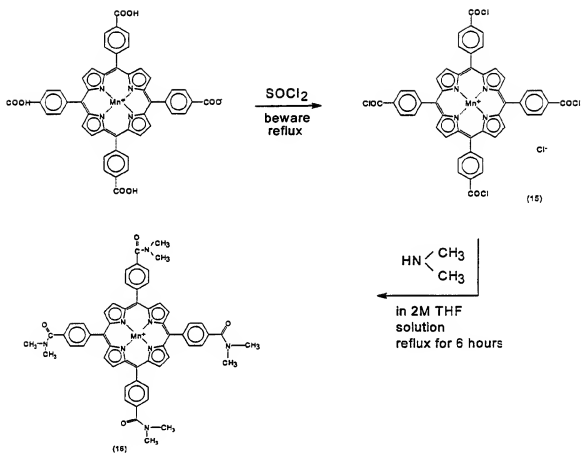
A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 38/44, 45/05; C12N 5/00, 9/02,15/00; C07H 21/04

US CL :424/85.1, 85.2, 94.4; 435/189, 240.2, 320.1; 536/23.2

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/85.1, 85.2, 94.4; 435/189, 240.2, 320.1; 536/23.2.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95/10185 A1 (DUKE UNIVERSITY) 20 April 1995, see the entire patent.	1-21, 24-43
X	Biochem. J., Volume 298, Issued 01 June, 1994, Stralin et al., " Effects of Oxidative Stress on Expression of Extracellular Superoxide Dismutase, CuZn-Superoxide Dismutase and Mn-Superoxide Dismutase in Human Dermal Fibroblast", pages 347-352. See abstract on page 347 and page 349 for Table 1.	17-19
X,P	US 5,472,691 A (MARKLUND et al.) 05 December 1995, column 40, claims 1-3.	19-20

☒ Further documents are listed in the continuation of Box C.	☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 AUGUST 1996	04 OCT 1996

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	TEKCHAND SAIDHA
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Genomics, Volume 22, 1994, Folz et al., " Extracellular Superoxide Dismutase (SOD3): Tissue-Specific Expression, Genomic Characterization, and Computer-Assisted Sequence Analysis of the Human EC SOD Gene", pages 162-171. Characterization of human EC SOD gene and its flanking regions. Partial nucleotide sequence and deduced amino acid sequence is disclosed on page 168, Fig.5.	24-25
X	US 5,248,603 A (MARKLUND et al.) 28 September 1993, Figs. 5a and 5b show the DNA sequence and deduced amino acid sequence of EC-SOD.	24-25
Y	American Journal of Respiratory Cell and Molecular Biology, Volume, 8, 1993, Clyde et al., " Distribution of Manganese Superoxide Dismutase mRNA in Normal and Hyperoxic Rat Lung", pages 530-537. See page 530, abstract, disclosing the tissue specificity of Mn-SOD in relation to hyperoxia in rat lungs.	27
X	US 5,366,729 A (MARKLUND et al.) 22 November 1994, see abstract, examples 1-8 for detailed description of the EC-SOD variants and the recombinant DNA methods to produce the variants.	19-20 and 24-25

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/10497

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such
   an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 22 & 23
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable
   claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment
   of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers
   only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is
   restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest   ☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(1))(July 1992)★

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

STN data base, files: CaPlus, Medline, WPIDS and Biosis. Registry file for structure search of chemical compounds. APS search. Search Terms used: Oxidant Scavenger?, superoxide dismutase, catalase, ec sod.